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Monitoring toxicity in raw water of the Cache la Poudre River and Sheldon Lake, Colorado, USA using biomarkers and molecular marker technology.

by

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Submitted in partial fulfilment of the requirements for the degree

Philosophiae Doctor

In the Department of Microbiology and Plant Pathology,
Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

December 2005

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Acknowledgements

I would like to thank the following people and institutions:

- Prof. T.E. Cloete for his guidance, support and advise during this study and preparation of this manuscript.
- Prof. A-M. Botha-Oberholster for suggestions, guidance and bearing with me during the course of the study.
- The Colorado State University for their facilities.
- The University of Pretoria; Department of Genetics for their facilities and materials provided.
- The Water Research Commision and National Research Foundation for funding this project.
- The National Research Foundation for the bursary provided.
- Leanne Coetzee, City Council of Tshwane and Karin van Ginkel, Department of Water Affairs and Forestry for providing research materials.
- Much appreciation to my sons, family and friends for their interest and support.

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DECLARATION

I the undersigned hereby declare that the work carried out in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Paul Johan Oberholster
20th December 2005

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List of Abbreviations

aa	Amino acid
ABS	Absorbed photon flux
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
APHA	American Public Health Association
ASTM	American Standard Test Method
AUSRIVAS	Australian river bioassessment system
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	Base pair
CCAP	Culture Collection of Algae and Protozoa, UK
Co	Company
CTAB	N-cetyl-N-N-N-trimethyl ammonium bromide
DAF	DNA amplification fingerprinting
dATP	Deoxyadenine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double distilled water
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DIN	Dissolved inorganic nitrogen
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynuclein triphosphate
DTE	Dithioerythritol
DTT	Dithiothreitol
dTTP	Deoxythymine triphosphate
dUTP	Deoxyuracil triphosphate
EC	Enzyme code
EDTA	Ethylenediamine tetra-acetic acid, disodium magnesium
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ET	Electron transport past Q_{A-}
e-value	expectancy value
F_0	Minimal fluorescence of a dark adapted sample
F_m	Maximal fluorescence of a dark adapted sample
GC	Gas chromatography
Hepes	4-(2-Hydroxyethyl)iperazine-1-ethanesulfonic acid
HPLC	High performance liquid chromatography
I_k,	the light intensity at the onset of light saturated photosynthesis in µmol photon m^{-2} s^{-1}
i.p.	intraperitoneally
IPTG	Isopropyl-β-D-galactoside

i.v.	intravenous
kb	Kilobase
kDa	Kilodalton
LB	Luria Bertrani
LD_{50}	Lethal dose
LDH	Lactate dehydrogenase
MC	Microcystin
Mdha	N-methyl-dehydroalanine
MI	Marker Index
MMPB	3-methoxy-2-methyl-4-phenylbutric acid
mRNA	Messenger ribonucleic acid
NBT	Nitroblue tetrazolium salt
NIES	National Institute for Environmental Studies, Japan
PAH	polycyclic aromatic hydrocarbons
P^B_{max}	maximum biomass specific photosynthetic rate in µmol O_2 mg chl a^{-1} h^{-1}
PCC	Pasteur Culture Collection
PCR	Polymerase Chain Reaction
PCR-RFLPs	Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms
PcoA	Principal coordinate analysis
PIC	Polymorphic Information Content
PP	Protein phosphatase
PPi	Inorganic pyrophosphate
RC	Reaction Centre
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SSC (20X)	0.3 M NaCitrate, 3 M NaCl, pH 7.0
SRP	soluble reactive phosphorus
STET	0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 5 % Triton®X-100
TAE (1X)	40 mM Tris-acetate, 1 mM EDTA, pH 8.0
TE	10mM Tris-HCl, 1 mM EDTA, pH 8.0
Tes	N-[Tris(hydroxymethyl)methyl]-2-aminoethane-sulfonic acid
TOC	Total organic carbon
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	Transfer ribonucleic acid
UP	University of Pretoria
UPGMA	Unweighted Pair Group Method using Arithmetic averages
UV	Ultraviolet
UV	Strain in the University of the Free State Culture collection
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-phosphate	Toluidinium salt

List of Units

LD_{50} Dose of toxin that kills 50 % of the animals tested.
LC_{50} Lethal concentration of toxin that kills 50% of the tested organisms.
LT_{50} Lethal time that toxin take to kill 50% of the tested organisms.

Restriction Enzyme

One unit is the enzyme activity that completely cleaves 1µg λDNA in 1 h at enzyme specific temperature in a total volume of 25 µL.

Taq DNA Polymerase

One unit is the quantity of enzyme required to catalyse the incorporation of 10 nmol of dNTP's into acid insoluble material in 30 minutes at 74 °C.

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CHAPTER 1

Introduction

Lakes, reservoirs and freshwater wetlands are a significant part of the Colorado landscape to such an extent that more than 1 500 lakes and reservoirs comprises about 66 368 hectares (~164 000 acres) in the state of Colorado (Colorado Department of Health and Environment, 2000). A recent survey by scientists of the water resources in Colorado, USA revealed the presence of several forms of pollutants present in streams and groundwater sources (Stevens 2003; also see http://co.water.usgs.gov, January 20, 2005) and thus, that the quality of Colorado's waters is deteriorating. The water resources in Colorado is affected by both natural and anthropogenic factors. The natural conditions include geological formations, the topographic and climatological factors controlling the hydrologic regime, and the density of vegetation within watersheds. Anthropogenic factors are mainly the result of discharges of pollutants, polluted runoff from various land uses and development in irrigation systems and floodplains (Stevens 2003).

The Cache la Poudre River in the South Platte River Basin serves as one of two surface-water sources (together with the Horsetooth Reservoir) for the city of Fort (Ft.) Collins in Larimy County, Colorado (Fig. 1.1). The Cache la Poudre River originates in the northern Colorado near the Continental Devide. The river flows out of the Rocky Mountain National Park, through the city of Fort Collins, and eventually into the South Platte River near Greeley, Colorado. Snowmelt provides most of the water to the river, with storm runoff in the summer months and ground-water inflow throughout the year (Collins & Sprague 2005). During the summer months, the Cache la Poudre River is supplying raw water to Sheldon Lake in Ft. Collins (Lake Drainage Improvement Project 2002, 2003a, b). Both these water bodies are extensively utilized

for recreation and irrigation purposes, while the Cache la Poudre River also provides for drinking water to the city of Ft. Collins (Collins & Sprague 2005).

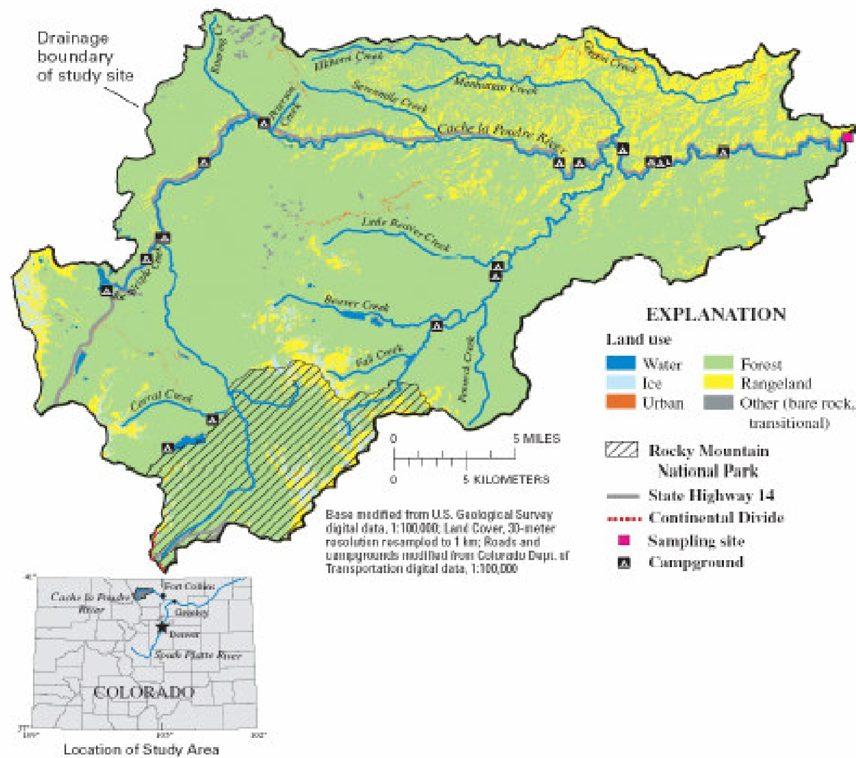

Figure 1.1 Map indicating the catchment area of the Cache la Poudre River (Adapted from Collins & Sprague 2005).

In my Ph.D. thesis, I proposed that anthropogenic activities have a negative impact on selected water resources in Colorado, USA. The main objectives of this study were (i) to investigate the impact of anthropogenic activities on two water bodies in Ft. Collins (e.g., Cache la Poudre River and Sheldon Lake); (ii) to gain scientific knowledge on management and restoration practices, and the effects thereof; and (iii)

to provide a fast, accurate, robust and relatively easy genetic/biological marker system for assessment of the water quality in water resources in general.

For my experimental model, I have selected two water resources in Ft. Collins, since the Cache la Poudre River is one of the main drinking water sources for near >200 000 residents of Larimy County (including Ft. Collins), Colorado. The city of Ft. Collins water-treatment facility produces more than 37.9 billion liters of treated water annually to serve the needs of the community's residents (Collins & Sprague 2005). The Cache la Poudre River is further the main supplementing water source to the urban Sheldon Lake during the summer months. In contrast to Sheldon Lake that is only receiving water as a drainage system, Cache la Poudre Rive is a free-flowing river system, that supplies water to several urban lakes and the South Platte River. Both aquatic ecosystems are model systems for the study of the impact of anthropogenic activity that necessitated restoration, and then recovery after restoration. Sheldon Lake was restorated prior to the study, while the Cache la Poudre River is presently under restoration with the support of the U.S. Environmental Protection Agency. Restoration of both water bodies was necessary due to poor water quality as a result of anthropogenic pollution.

The chapters of the thesis are independent units by themselves; however, they converge towards addressing more or less the impacts of anthropogenic activity on aquatic ecosystems, using the above two study areas, it further use biomarkers and molecular markers to assess raw water health and the dynamics and diversity in the aquatic ecosystem.

Outline of Chapters

Chapter 2

In Chapter 2, I give a review on the existing information relating to the impact of anthropogenic activity (e.g., urban run-off, eutrophication) on the quality of water resources in general and specific to the US. Toxic chemical pollutants and cyanobacterial toxins and the risk posed by these compounds to human health are discussed. Economic losses related to the presence of freshwater pollutants are the result of contact with or consumption of water containing toxic substances and/or toxic biological organisms/cells, and these include the costs incurred from death of domestic animals, allergic and gastrointestinal problems in humans and water treatment. I further discuss applied technology for assessment of aquatic health, which includes: the use of biological organisms as bioindicators and other detection methods, specifically the use of PCR-based technology that has a great potential as a fast screening method to detect toxic cyanobacteria strains in water bodies.

Chapter 3

In Chapter 3, I have examined the impact of long-term contamination of coal tar residue on benthic organisms in the Cache la Poudre River, Colorado. Hazardous chemicals such as oils and hydrocarbons are common water pollutants generated from a variety of industrial processes, such as oil refineries, coke ovens and coal gasification. Toxicity of oils to organisms is usually related to their content of non-volatile aromatic hydrocarbons (Zikto & Carson 1970; Neff & Anderson 1975).

Hufford (1971) revealed that the most immediate toxic and subtoxic fractions of oils are those soluble in water. Knowledge of the effects of oil spills on freshwater ecosystems is limited compared to the wealth of information available on oil entering the marine environment. In the United States, 179 freshwater oil spills were reported between 1979 and 1986, and spills exceeding 200 000 L occurred 25 times between 1974 and 1980 (Cronk *et al.* 1990). Petroleum releases into the aquatic environment are a leading cause of fish kills in the United States (Green & Trett 1989). Crunkilton (1984) indicated that one-third of all reported water pollution incidents in Missouri were petroleum-related.

The first objective of Chapter 3 was to apply the Australian river bioassessment system (AUSRIVAS) on a long-term coal tar contaminated area of the Cache la Poudre River, Colorado. This method is based on the presence and absence of aquatic macroinvertebrates, and compares the numbers and types of organisms found at a site with those predicted to be present on the basis of a model derived using data from minimally degraded reference sites. The second objective was to use several test species, belonging to different trophic levels as biomarkers to provide rapid toxicity assessment of the coal tar contaminated area of the Cache la Poudre River. Since the Cache la Poudre River is supplying water to Sheldon Lake, its water quality will be a determining factor in the general "health" of the aqua ecosystem in the urban Sheldon Lake (see Chapters 4 and 5).

Chapter 4

The goal of chapter 4 was to assess the ecological status, as well as the limnological condition of Sheldon Lake a year after restoration. Succession is one of the more obvious and regular features of a lake. Hence, some organisms always occur during spring, whereas others exclusively dominate during summer or in late autumn, forming a succession pattern that is generally repeated every year. Factors that may be affecting seasonal succession are the trophic state of a lake, the different physical or chemical requirements of organisms, including temperature and nutrient or food preferences. Interactions among the organisms themselves are also important, including predation, grazing and competition pressure. Hence, following a seasonal cycle in Sheldon Lake will reveal the abiotic as well as biotic processes acting to shape the observed succession pattern among organisms after restoration.

Chapter 5

The purpose of the study in Chapter 5 was to evaluate the efficacy of artificial mixing in controlling cyanobacterial bloom formation in summer during low wind velocity. In chapter 5, the use of PCR amplification of the *mcy* gene cluster in environmental *Microcystis aeruginosa* samples of Sheldon Lake for early detection of potentially toxic *Microcystis* mass occurrences a year after restoration of this shallow urban lake was assessed. Since microcystins are synthesized nonribosomally by a peptide synthetase polyketide synthase enzyme complex encoded by the microcystin synthetase (*mcy*) gene cluster (Christiansen *et al.* 2003; Dittmann *et al.* 1997; Nishizawa *et al.* 1999, 2000; Tillett *et al.* 2000). Further objectives were to use PCR amplification of the *mcy* gene cluster to detect the toxicity through gene expression of

Microcystis populations. By doing so it will provide us with more information on the microcystin producing genotypes that form the epilimnetic population.

Chapter 6.

In Chapter 6, I investigated the usefulness of a novel DNA fingerprinting technique, AFLP, which is based on the selective amplification of genomic restriction fragments by PCR, to differentiate between geographical unrelated strains of *Microcystis spp.* because some species may occur more widely, but with morphologies that are phenotypically uniform in one area and variable in another. Examples of variable cyanobacteria populations in comparison with other regions are *Woronichinia naegeliana* in Canadian lakes (Komárek & Komarkova-Legnerova 1992) and *Microcystis wesenbergii* in Northern Europe (Cronberg & Komárek 1994). Komárek and Anagnostidis (1989) stated that the feature of more than 50% of the strains in collections do not correspond to the diagnoses of the taxa to which they are assigned.

Chapter 6 further aims to supply insight into the genetic diversity of the newly collected toxic strains in the study (i.e., UPUS1 *M. aeruginosa* and UPUS2 *Woronichinia naegeliana*), since little information is available on Colorado cyanobacterial strains in general. The study, in part, aims to put the Colorado strains in genetic "context" to other reference strains used during the study (i.e., *M. aeruginosa* PCC7806 and *M. aeruginosa* UP37). Both strains tested toxic using ELISA and PP2A methodology, with UP37 the most toxic strain collected in South Africa during the study (see Chapter 5).

Finally, I am presenting a summary of all the observations made through the study. I am also concluding with recommendations with regard to restoration and management practices (see Summary).

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CHAPTER 2

An overview of the impact of anthropogenic activity on water quality with special reference to human health, risks and detection of toxic compounds present in water bodies.

A selection from the contents this chapter has been published in *Biochemistri* 17(2): XXX (2005)
(Manuscript submission number: BKM 2005/024; Date of acception: October 22, 2005)

University of Pretoria etd – Oberholster, P J (2006)

2.1 Introduction

For most of history, human population and the level of technological development have been too low to drastically affect the global quality of air, soil, water and biological systems. However, with the world's population increasing to 6 billion, pressures on the environment, and especially the water resources is increasing. Approximately, 65% of the 1015 billion litres of freshwater withdrawn for all purposes in the United States in 1985 was obtained from surface water sources (Solley *et al*. 1988). While water quality is generally good in the United States, many municipal water supply systems are operating potentially harmful contaminant and pollution levels. In 1976, it was estimated that some 8 million US inhabitants were using potentially dangerous water from about 5 000 community supplies (U.S. Environmental Protection Agency, 1976), and with numerous reports of illnesses due to contamination of drinking water resources (see later Table 2.2), the importance of water quality is highlighted.

Anthropogenic disturbances change the physicochemical characteristics and resource availability of rivers from headwaters to mouth and influence community structure and function of stream biota. Assessment of stream system health cannot be fully achieved without a careful analysis of the benthic fauna and benthic processes (Reice & Wohlenberg 1993). Macroinvertebrates and phytoplankton are a critical component of every stream. They play an essential role in the food chain, productivity, nutrient cycling, and decomposition (Cummins 1988). Knowledge of the state of the macroinvertebrate community is, therefore, essential to the assessment of the health of the stream system.

2.2 Water quality

Water serves as the universal medium for all metabolic reactions, and life cannot exist without water. The quality of surface water is currently arousing considerable interest, due to the importance of this resource for human life and activities (U.S. Environmental Protection Agency 2004). Aquatic pollutants originate from point sources and/or from non-point sources. The former comprises effluents from sewers originating from human settlements or industries where one can easily identify the source of pollution. The non-point sources include surface runoff laden with agricultural chemicals such as pesticides and fertilizers. Domestic sewage disposal and urban runoff causes the most serious water pollution problems in the more densely populated areas throughout the world. Domestic sewage mainly increases biological oxygen demand and nutrient levels of the receiving waters (Mason 1996). Progressive deterioration of many surface water ecosystems has been reported all over the world. In the United States alone, one of every three lakes and nearly one-quarter of the nation's rivers contain enough pollution that people should limit or avoid eating fish caught there. Every state but Alaska and Wyoming issued fish advisories covering some and occasionally all of their lakes or rivers in 2003, according to a national database maintained by the U.S. Environmental Protection Agency (U.S. EPA 2004). A recent survey in 2002-2003, assessing the water quality of the Cache la Poudre River, Colorado, revealed the presence of pesticides; wastewater compounds and *Esterichia coli* bacteria; dissolved organic compounds; 89 volatile organic compounds and polycyclic aromatic hydrocarbons (PAHs) (e.g., coaltar residues, acetone, toluene and benzene) (Collins & Sprague 2005).

2.3 Urban runoff

Urbanization is inevitably associated with the greatly increase runoff problems due to the fact that roads, parking lots, rooftops and other such surfaces are impervious to water. Rainfall that lands on these areas has no chance to sink into the ground, as would be the case if the land were covered with vegetation. The percentage of land cover by impervious surface in business districts may approach 100% and in residential areas may be easily 50%. Thus, the amount of overland runoff from urban areas is much greater than in comparable rural areas. Furthermore, runoff from impervious surfaces is more rapid than from land covered with vegetation, since there is little to impede the flow of water over parking lots, streets, sidewalks or urban areas than from rural areas. Because the erosive power of water increase with the intensity of water flow and because there is more total runoff in urban areas, exposed land is eroded away more rapidly in urban than in rural areas. The crux of the problem is the fact that in many communities urban runoff, which may contain high concentrations of nutrients, oxygen-consuming wastes, pathogens and toxic substances such as pesticides, heavy metals, and oil is simply routed to the nearest convenient watercourse and discharged into a stormwater management pond or detention lake without treatment (Peterson *et al.* 1985). The limited available data on the presence of pollutants in urban runoff indicate that urban streams are the most vulnerable to contamination, with one or more wastewater chemicals found in 100% of the samples collected and tested from urban streams in Colorado (Sprague & Battaglin 2004).

2.4 Eutrophication

Nutrient pollution especially with phosphorus but also with nitrogen coming from urban runoff and sanitary sewer systems can lead to the eutrophication of the receiving water bodies (Stevens 2005). Eutrophication of water bodies has increased during the last century due to an increase in anthropogenic inputs. In Europe, Asia and America, more than 40% of lakes are now eutrophic and hence subject to algal proliferations (Bartram *et al*. 1999). The perturbation of the natural succession of phytoplankton in eutrophic and especially hypertrophic waters extends and intensifies the period of cyanobacterial dominance. Cyanobacterial water blooms can degrade water quality in many ways. A high cyanobacterial biomass can contribute to aesthetic problems such as scum formation that dramatically decreases the transparency of the water body and can also cause the production of undesirable tastes and odours in drinking water (WHO 1998). From an economic standpoint, the most important problem relates to taste and odours in drinking water, and the potential for this to happen in the US is high due to the fact that more water is utilized from surface water than ground water (Solley *et al*. 1988). During the decomposition of water blooms, deoxygenation of waters is observed which can affect the ability of aquatic animals to survive. One of the most serious effects of bloom formation can be the production of secondary metabolites by cyanobacteria, which can be toxic to humans and animals. In 1998, risks to humans, lead the World Health Organization (WHO) to propose a provisional guideline value of 1.0 μg/L for the levels of one of the most common cyanotoxins, the microcystin-LR in drinking water (WHO 1998).

Toxins of cyanobacteria are grouped in two main categories by Carmichael (1992) namely, biotoxins and cytotoxins based on the types of bioassays used to screen for

their activity. The presence of cytotoxins is detected by mammalian cell lines and biotoxins are assayed with small animals, e.g. mice or aquatic invertebrates. The primary types of cyanobacterial biotoxins include hepatotoxin (microcystins, nodularins, cylindrospermopsins), neurotoxin (anatoxins, saxitoxins) and dermatotoxins (lyngbyatoxin A, aplysiatoxins, lipopolysaccharides) (Codd & Poon 1988; Codd & Bell 1989), and in the toxicity standards, biotoxins are considered supertoxic (Table 2.1). Biotoxins of cyanobacteria are water-soluble and heat stable and they are released upon aging or lysis of the cells. The most frequently occurring hepatotoxins are microcystins. They are cyclic peptides of molecular weight from about 800 to 1 100. They contain seven amino acids. More than 60 different structural variations of microcystin have been characterized so far (Burns *et al*. 2004). The majority of microcystin variants differ by L-amino acids in positions 2 and 4. For microcystin-LR, the two variable amino acids are leucine and arginine that have the single letter abbreviations L and R, respectively (Codd & Poon 1988; Carmichael 1992).

Table 2.1 Comparison of toxicities of some biological toxins

Toxins	Sources	Lethal doses (LD_{50})	Reference
Saxitoxin	*Aphanizomenon flos-aquae*	10	(Oshima 1995).
Anatoxin-a(s)	*Anabaena flos-aquae*	20	(Falconer 1998).
Cobra toxin	*Naja naja*	20	(Bagchi 1996)
Nodularin	*Nodularia spumigena*	30	(Rinehart *et al*. 1994).
Microcystin LR	*Microcystis aeruginosa*	50	(Rinehart *et al*. 1994).
Anatoxin-a	*Anabaena flos-aquae*	200	(Carmichael 1992).
Brevetoxin	*Karenia brevis (dinoflagellate)*	500	(Morohashi *et al*. 1999).
Ciguatoxin	*Gambierdiscus toxicus (dinoflagellate)*	0.25	(Bagnis *et al*. 1980).
Cylindrospermopsins	*Cylindrospermopsins raciborskii*	2 100	(Ohtani *et al*. 1992).
Styrchnine	*Strychnos nuxvomica*	2 000	(Bagchi 1996).

Toxic cyanobacteria have caused mortalities amongst wild and domestic animals (Codd 1992). They also constitute a hazard to human health, for example, via ingestion and skin contact (Carmichael 1992). The first clearly documented human fatalities, which are ascribed to cyanobacterial toxins, occurred in 1996. More than 50 patients at a hemodialysis center in Caruaru died with hepatotoxic and neurotoxic symptoms (Pouria *et al*. 1998). Long-term chronic exposure of humans to cyanobacterial toxins may also occur, as some conventional water treatment processes are ineffective in the removal of toxins from drinking water (Himberg *et al*. 1989). The cyanobacterial hepatotoxins have also been shown to have tumor-promoting activity and can lead to primary liver cancer (Fujiki 1992). Cyanobacterial blooms are not always toxic. At the same sampling point, it is possible to find toxic and nontoxic strains of the same species of cyanobacteria.

2.5 Human health risks of long-term exposure to toxic compounds

2.5.1 Health risks of long-term exposure to low levels of microcystin

Little information is available on the effects of long-term exposure to low levels of microcystin toxins in humans (Table 2.2). We know that in experiments performed on a time-scale of minutes or hours, microcystin has obvious effects on the functions of plant and animal cells at concentrations as low as 3-10 nM that is equivalent to 3-10 µg for an adult female liver. In cells that take up microcystin freely, the maximum effects are visible at concentrations of around 1µM, the point at which all of the cellular PP1 and PP2A is saturated with toxin. This means that approximately 1 mg (equivalent to drinking to two liters of water per day at 32 µg/L microcystin over two weeks) would bind all of the PP1 and PP2A in an adult female human liver, provided

that the PP-microcystin complexes were stable (MacKintosh 1993). However, most of the available data about uptake and turnover of microcystins has been obtained from experiments carried out with rodents. In this regard, it should be noted that PP1 and PP2A from mice and humans amino acid sequences are 100 percent identical (Barker *et al.* 1993). In the case of mice low doses of microsystin cause progressive changes in liver tissue over time, including chronic inflammation, focal degeneration of hepatocytes and the accumulation of metabolites such as bilirubin in the blood, and tend to increase mortality (Hermansky *et al.* 1990). In South Africa liver damage and death of vervet monkeys has occurred following toxic *Microcystis* administration with signs of poisoning similar to those observed in live stock and mice (Tustin *et al.* 1973). These demonstrations of the susceptibility of primates to cyanobacterial poisoning are consistent with the results of an epidemiological study of a human population of the city of Armidale, New South Wales, Australia, which obtains its drinking water from the Malpas Dam reservoir. A clear pattern of admission of patients to the local hospital with liver complaints was identified which coincided with the seasonal production of a hepatotoxic *Microcystis aeruginosa* bloom in the reservoir. This correlation was confined to patients who had taken their drinking water from the Malpas Dam (Falconer *et al.* 1983).

Yu (1995) reported that the incidence of liver cancer is significantly higher for populations using cyanobacteria-infested surface water than those drinking groundwater in China. In Shanghai and its nearby regions where epidemiological studies showed that increased incidence of primary liver cancer is related to the consumption of microcystin contaminated water, the concentrations of microcystins in samples of pond-ditch water were within the range of 0.09-0.46 µg/L (Ueno *et al.*

1996). However, Zegura *et al*. (2002) showed that microcystin-LR induced oxidative DNA damage in HepG2 human cells at low concentrations (0.01 μg/mL) and this might be a mechanism by which chronic exposure to low concentrations of microcystins contribute to increase the risk for liver cancer development. A recent study in mice has shown that *Microcystis aeruginosa* extract provided in drinking water increased the area of aberrant crypt foci in the colon, suggestive that microcystins promote preneoplastic colonic lesions (Humpage *et al*. 2000a).

Table 2.2. Acute intoxications of humans from cyanobacteria.

	Cases attributed to cyanotoxins in drinking water
Year	**Report**
1931	United States; A massive *Microcystis* bloom in the Ohio and Potomac rivers caused illness in 5 000 to 8 000 persons whose drinking water was taken from these rivers. Low rainfall has caused the water of a side branch of the river to develop a cyanobacterial bloom, which was then washed by new rainfall into the main river. Drinking water treatment by precipitation, filtration, and chlorination was not sufficient to remove the toxins (Tisdale 1931; Veldee 1931).
1960-1965	Zimbabwe, Harare; Cases of acute gastroenteritis among European children admitted to the local hospital in Salisbury, Rhodesia (now Harare, Zimbabwe). In this instance, several supply reservoirs provided water to different regions of the city, but only the reservoir containing blooms of *Microcystis* supplied water to the affected population. (Zilberg 1966).
1968	United States; Numerous cases of gastrointestinal illness after exposure to mass developments of cyanobacteria was compiled by Schwimmer and Schwimmer (1968).
1975	United States; Hindman *et al*. (1975) reported the results of an investigation into 49 pyrogenic reactions in patients undergoing haemodialysis treatment in Washington, DC. They concluded that 'the cause of these reactions was traced to an increase in endotoxin contamination of the tap water used to prepare dialysate, possibly caused by an increase in the algae levels in the local water source.
1979	Australia; Combating a bloom of *Cylindrospermopsis raciborskii* in a drinking water reservoir on Palm Island with copper sulfate led to liberation of toxins from the cells into the water, thus causing serious illness with hospitalization of 141 persons supplied from this reservoir (Falconer 1993a, 1993b).
1981	Australia; In the city of Armidale, liver enzyme activities were elevated in the blood of the population that was supplied from surface water polluted by *Microcystis* spp. (Falconer *et al*. 1983).
1992	United States; Carmichael (1992) compiled case studies on nausea, vomiting, diarrhea, fever and eye, ear, and throat infections after exposure to mass developments of cyanobacteria.
1993	Australia; Ressom *et al.* (1994) estimated that more than 600,000 person-days are lost annually due to absence of their water source due in turn to toxic cyanobacterial blooms.
	China; The incidence of very high rates of liver cancer is related to water sources. The incidence is significantly higher for populations using cyanobacteria-infested surface waters than those drinking ground water. A cohort study showed that people who drank pond and ditch water had 121 deaths per 100 000 compared with 0 for those who drank well water (Yu 1994, 1995).
1994	Sweden Near Malmo; Illegal use of untreated river water in a sugar factory led to an accidental cross-connection with the drinking water supply for an uncertain number of hours. The river water was densely populated by *Planktothrix agardhii*, and samples taken a few days before and a few days after the incident showed these cyanobacteria contained microcystins. Of 304 inhabitants of

	the village, 121 became ill with vomiting, diarrhea, muscular cramps, and nausea (Cronberg *et al*. 1995).

Date	**Cases attributed to cyanotoxins in recreational water** **Description**
1959	Saskatchewan, Canada; In spite of livestock deaths and warnings against recreational use, people did swim in a lake infested with cyanobacteria. Thirteen persons became ill (headaches, nausea, muscular pains, painful diarrhea). In the excreta of one patient – a medical doctor who had accidentally ingested 300 ml of water-numerous cells of *Microcystis* spp. And some trichomes of Anabaena circinalis could be clearly identified (Dillenberg & Dehnel 1960).
1989	England; In Staffordshire ten out of 20 soldiers became ill after swimming and canoe-training in water with a heavy bloom of *Microcystis* spp.; two of them develop severe pneumonia attributed to the inhalation of a *Microcystis* toxin and required hospitalization and intensive care. Sixteen develop sore throat, headache, abdominal pain, dry cough, diarrhoea, vomiting and blistered mouths (Turner *et al*. 1990). Swimming skills and the amount of water ingested appear to have been related to the degree of illness.
1995	Australia; Epidemiological evidence of adverse health effects after recreational water contact from a prospective study involving 852 participants who showed elevated incidence of diarrhea, vomiting, flu symptoms, skin rashes, mouth ulcers, fevers, and eye or ear irritations within 2 to 7 days after exposure. The sensitivity of individuals to allergic-type reactions at low cyanobacteria cell densities is greater than can be attributed to the toxin content of cyanobacteria (Pilotto *et al*. 1997).

Date	**Cases due to other exposure routes** **Description**
1996	Caruaru in Brazil; One hundred and twenty six dialysis patients were exposed to microcystin through the water used for dialysis, and 60 of them eventually died, principally of liver failure, 6 had died by 2 weeks after exposure, 30 by 6 weeks, 44 by 10 weeks, and 55 by 27 weeks. At least 44 of these victims showed the typical common symptoms associated with microcystin, now referred to as 'Caruaru Syndrome' and the liver microcystin content corresponded to that of laboratory animals that received a lethal dose of microcystin (Jochimsen *et al*. 1998; Carmichael *et al*. 1996; Pouria *et al*. 1998).

2.5.2 Effect of long-term exposure to PAHs

Petroleum products like PAHs can adversely affect organisms by physical action, habitat modification and toxic action. The aromatic fraction of petroleum is considered to be responsible for most of the toxic effects, thus PAHs affect organisms through toxic action. The mechanism of toxicity is reported to be interference with cellular membrane function and enzyme systems associated with the membrane (Neff 1985). Although unmetabolized PAHs can have toxic effects, a major concern in animals is the ability of the reactive metabolites, such as epoxides and dihydrodiols of some PAHs to bind to cellular proteins and DNA. The resulting biochemical

disruptions and cell damage lead to mutations, developmental malformations, tumors, and cancer (Eisler 2000; Santodonato *et al*. 1981; Varanasi 1989). Four-, five-, and six-ring PAHs have greater carcinogenic potential than the two-, three-, and seven-ring PHAs (Eisler 2000; Neff 1985). The addition of alkyl groups to the base PAH structure often produces carcinogenicity or enhances existing carcinogenic activity. Some halogenated PAHs are mutagenic without metabolic activation (Fu 1999) and the toxicity and possibly the carcinogenicity of PAHs can be increased by exposure to solar ultraviolet radiation (Ren 1994; Arfsten *et al*. 1996). Cancerous and precancerous neoplasms have been induced in aquatic organisms in laboratory studies, and cancerous and noncancerous neoplasms have also been found in feral fish from polluted sites (Eisler 2000; Neff 1985; Baumann 1989; Chang *et al*. 1998).

2.6 Methods for detection of toxicity as a result of chemical pollutants and biological toxic compounds in raw water

2.6.1 Bioassays for detection of PAHs

In recent years the increasing desire to link exposure, to effect has drawn considerable attention to the ‘biomarker approach’. Because chemical contaminants are known to evoke distinct measurable biological responses in exposed organisms, biomarker-based techniques are currently being investigated to assess toxicant-induced changes at the biological and ecological levels (Shugart 1996). Collectively the term biomarker refers to the use of physiological, biochemical, and histological changes as ‘indicators’ of exposure and effects of xenobiotics at the suborganism or organism level (Huggett *et al*. 1992).

However, indicators or biomarkers can be defined at any level of biological organization, including changes manifested as enzyme content or activity, DNA adducts, chromosomal aberration, histopathological alterations, immune-system effects, reproductive effects, physiological effects, and fertility at the molecular and individual level, as well as size distributions, diversity indices, and functional parameters at the population and ecosystem level. In the field of ecotoxicology, the use of biomarkers has emerged as a new and powerful tool for detecting both exposure and effects resulting from environmental contaminants (Huggett *et al.* 1992; Shugart *et al.* 1989, 1992a, 1992b; McCarthy & Shugart 1990; Peakall & Shugart 1992; Fossi & Leonzio 1993; Travis 1993). Unlike most chemical monitoring, biomarker endpoints have the potential to reflect and assess the bioavailability of complex mixtures present in the environment as well as render biological significance. Biomarkers provide rapid toxicity assessment and early indication of population and community stress and offer the potential to be used as markers of specific chemicals. In the European Inventory of Existing Commercial Substances (EINECS) about 100,000 chemicals are identified, of which approximate 10-20 chemicals are monitored in important European aquatic ecosystems. If a closer look is taken at the effects data for the chemicals in the EINECS, it appears that only for 20-30 chemicals adequate information is available on long-term single-species ecotoxicity and environmental fate. Hence, for 99.99% of all existing chemicals no adequate information on sources, effects and concentrations is available, which implies the importance of biomarker assessment on chemicals like coal tar residue (Van Leewen 1992).

2.6.2 Methods for detection of microsytin

The most common methods for monitoring microcystin concentrations have been high-performance liquid chromatography combined with a UV-visible light diode array detector, protein phosphatase inhibition and enzyme-linked immunosorbent assays (ELISA) (Harada *et al*. 1999). The intraperitoneal mouse bioassay, which has been the most extensively used biotest to determine the toxicity of cyanobacterial blooms, is useful as an initial screening test, but requires expensive husbandry, is strictly regulated in some countries, and is opposed on moral grounds (Bell & Codd 1996). However, such analysis does not indicate which cyanobacterial strains produce the toxins. Microcystin concentration in a body of water seems to be mostly dependent on the density of the hepatotoxic cells (Sivonen & Jones 1999). It has also been demonstrated that some strains may produce higher concentrations of microcystins than other strains under the same laboratory conditions. In addition, environmental factors, such as nutrient concentrations, light, and temperature, may also affect the intracellular microcystin concentration (Sivonen & Jones 1999). Since it is not possible to distinguish toxic and nontoxic strains with a microscope, microscopic analysis cannot be useful in estimating toxicity of cyanobacterial strains.

2.6.2.1 Alternative Bioassays

Under cyanobacterial bloom conditions the amount of zooplanton is known to decrease (Lightner 1978), consequently, bioassays have been devised using *Daphnia* sp. and *Artemia salina* (Kiviranta *et al.* 1991). Major difficulties in the use of alternative bioassays, such as those based on *Artemia salina* are highlighted by Kiviranta *et al.* (1991). High to moderate concentrations of neuro-and hepatotoxins

can be detected in bloom samples using this organism, but mouse bioassay is more reliable. In contrast, a laboratory-grown strain of *Oscillatora agardhii* was toxic to larvae of mosquito *Aedes aegyptii* (Kiviranta & Abdelhameed 1994), *Artemia salina* and *Daphnia pulex*, but non-toxic to mice (Reinikainen *et al*. 1995). Also, toxicity differs for the larval and adult stages of the shrimp (*Artemia salina*), particularly for neurotoxins. It is possible that some compounds may enable the toxin to enter the larvae by affecting the biochemistry of the crustacean or by hydrogen bonding to the chelates, which are more easily absorbed. Therefore the use of alternative bioassays must be approached with caution (Kiviranta & Abdelhameed 1994).

2.6.2.2 Use of animal and/or cell bioassays

Fish health assessment or pathology usually deals with the causes processes and effects of disease, which is a function of the general health of the associated environment. Pathological investigations include procedures such as necropsis, histological examinations, parasitological examinations and liver enzyme assays (Albert &Washuta 1992).

Worldwide the mouse bioassay is the recognized standard in terms of establishing the LD_{50}, symptoms and effects of cyanotoxins, and thus by mouse bioassay the toxicity of cyanobacterial may be assessed (Chaivimol *et al.* 1994). Toxic symptoms vary depending upon material and concentration, but are in general distinct for a category of toxins. The symptoms of death of the animal allow for a clear distinction to be made as to whether the toxicity is due to a neuro or hepatotoxin. Neurotoxicosis is rapid compared to hepatotoxicosis and does not cause liver damage to animals (Falconer & Yueng 1992). A major disadvantage of the mouse bioassay is that it

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cannot detect low amounts of toxins and can therefore only be used for concentrated cell samples or concentrated toxin. In addition, it cannot distinguish between different types of neuro- or hepatotoxins, particularly when several are present in the same sample (Carmichael 1992).

Hepatotoxins cause deformation and ultrastructural changes of the cytoskeleton of mouse liver hepatocytes primarily due to the inhibition of phosphatase activity (Eriksson *et al*. 1990). Aune and Berg (1986) observed these changes and proposed that freshly prepared rat hepatocytes can be used to study the toxicity of cyanobacterial blooms. They have shown that the *in vitro* toxicity to cells can be correlated with control animal experiments when crude cyanobacterial biomass is used. Similar effects have been observed with permanent cell lines and erythrocytes (Grabow *et al*. 1982). However, despite the remarkable toxic potential of hepatotoxins *in vivo*, no cell lysis, liberation of lactate dehydrogenase or haemolysis has been observed after application of pure hepatotoxin to primary or permanent cell lines (Eriksson *et al*. 1987). Furthermore, mouse bioassays is also not always feasible, since standardized laboratory facilities with specific ratings is required by law, and thus, not suitable for testing by water purification plants.

2.6.2.3 Enzyme-linked assays

The discovery that the hepatotoxic cyanobacterial toxins produce their toxic effects through the inhibition of protein phosphatases 1 and 2A has laid the foundation for toxin assays based on the inhibition of these enzymes (Sim & Mudge 1994). This is a cheap, effective assay and reliable means of detecting all hepatotoxic cyanobacterial

toxins and has been enhanced by the development of a colorimetric procedure (Ward *et al*. 1997), provided the measurement of toxicity is the only requirement. However, it does not provide for high sensitivity and the specificity with regard to toxic peptides as with the enzyme-linked immunosorbent assay (ELISA) and reversed-phase high performance liquid chromatography (RP-HPLC) analysis.

An ELISA assay has been developed by Chu *et al*. (1989), to the point where ng/mL of microcystin can be quantitated in supplies of domestic water. This immunoassay is based on polyclonal antisera raised in rabbits against bovine serum albumin conjugated to microcystin-LR (Chu *et al*. 1989). The antisera showed good cross-activity with microcystin-RR, -YR, -LR and nodularin, but less with -LA and -LY. For detection of binding to the antisera the enzyme horseradish peroxidase was conjugated to microcystin-LR. The sensitivity of the immunoassay showed approximately 50% binding of the enzyme at a toxin concentration of 1 ng/mL, which is ideal for normal water quality testing. Although ELISA is easy, relative robust to use and provide for fast results indicating trends, it lacks sensitivity with regard to specificity of toxins (Echols & Jones 2005). The assay is also relatively expensive and requires a reasonablely well equipted laboratory (e.g., plate reader), which make it less suitable for small-scale water purification plants.

2.6.2.4 Chromatographic Analysis

Methods based on the chemical structure of hepatotoxin were developed for high sensitivity and specificity during analysis, these include chromatographic methods and atom bombardment and proton nuclear magnetic resonance technology. Initially

to purify the hepatotoxins, thin-layer chromatography was used, however, the technique lacked sensitivity and specificity, and thus, it was superceded by high performance liquid chromatography (HPLC). Since then HPLC was shown to be the method of choice for detection of toxins, hepatotoxins from several cyanobacterial species and environmental biomass with high sensitivity (Harada *et al*. 1996a,b). Resolution of the toxic fraction from *Microcystis aeruginosa* PCC7806 into a closely related peptide has been achieved by reversed-phase (RP) HPLC (Cremer & Henning 1991). Integration of diode array spectroscopy with RP-HPLC has provided a rapid and sensitive method of assessing the nature and concentration of toxic peptides from cyanobacterial blooms, and is probably the method of choice when high sensitivity and resolution is required (Echols & Jones, 2005).

The characteristics of the structure of hepatotoxins have been ascertained primarily by fast atom bombardment mass spectrometry (MS) and proton nuclear magnetic resonance (Kusumi 1996). More recently, matrix assisted laser desorption ionisation mass spectrometry has permitted procedurally easier analysis of cyanobacterial biomass. This presents the possibility of rapid, sensitive analysis of environmental biomass for the presence of cyanotoxins (Chaivimol *et al*. 1994). Although RP-HPLC and MS provide for high resolution distinction of toxic peptides, it is costly, time consuming and usssually applied for single preparative scale runs.

2.6.2.5 Monitoring toxigenicity of cyanobacterial strains by molecular assay

Monitoring the quality of water destined to public supply includes identification of potentially toxic cyanobacteria and their population density. Identification of such

microorganisms based on morphological features only, though widespread, has proven problematic, mainly for the genus *Microcystis*, due to its extensive phenotypic plasticity (Kondo *et al*. 2000).

Identification of a cyanobacterial genus by microscopic morphology or molecular analysis does not indicate the potential for toxin production. Different strains of one species can be morphologically identical but differ in toxigenicity. *Microcystis aeruginosa* for example has both toxic and nontoxic strains (Meiβner *et al*. 1996). There have been numerous attempts to refine the identification of strains by using specific gene analysis. Examples include the use of PCR-based methods for amplification of the phycocyanin intergenic spacer (PC-IGS) between the α- and β-subunits of the phycocyanin operon in environmental samples (Baker *et al*. 2001) the 16S-23S rRNA internally transcribed spacer region (Otsuka *et al*. 1999) and the DNA-dependent RNA polymerase (*rpoC*I) gene (Fergusson & Saint 2000). Although these molecular techniques have improved the accuracy of strain identification, they have not been able to distinguish toxigenic from nontoxigenic strains of the same species.

The biosynthetic pathway for production of microcystin has now been elucidated (Tillett *et al*. 2001) and this has enabled the development of specific oligonucleotide primers for gene common to production of microcystins (Tillett *et al*. 2001). To better detect microcystin-producing cyanobacterial strains, Neilan *et al*. (1999) and Nishizawa *et al*. (1999) have developed genetic probes directed, respectively, to the *mcyB* gene and to adenylation domains within the microcystin synthetase gene cluster. The *mcy* gene cluster contains 55kb of DNA encoding six large open reading frames,

mcyA-E and -*G*, together with a further four small open reading frames *mcyF* and *H-J*, placed in the chromosome (Tillet *et al*. 2001). The insertional inactivation of microcystin peptide synthetase gene *mcyB* of a *Microcystis aeruginosa* strain (PCC 7806) resulted in loss of microcystin production, showing their involvement in microcystin synthesis. It was also observed by Dittmann *et al*. (1997) that all isoforms of the cyclic heptapeptide were disrupted by inactivation of the microcystin synthetase gene sequence *mcyB*.

Several reports on the utility of the *mcyB* gene sequence for identification of toxic potential in environmental strains was published (do Carmo Bittencourt-Oliveira, 2003; Oberholster 2004; Grobbelaar *et al*. 2004; Grobbelaar 2005; Ouahid *et al*. 2005). Ouahid *et al*. (2005) applied PCR primers that were designed from the six characteristic segments of the microcystin synthetase *mcy* cluster to decern between toxin and non-toxin producing strains using ~2 000 cells as template in a multiplex reaction procedure. In a study by Oberholster (2004) and Grobbelaar *et al*. (2004), the *mcyB* gene from PCC7813 and UV027 were sequenced, resulting in fragments of 2174 and 2170 base pairs in size, respectively. The obtained sequences showed homology to other published sequences in GenBank (AY034601 for PCC7813 and AY034602 for UV027; e-value = 0.0). Upon further analysis of the sequences, it was obvious that there are several base differences between the sequences of the two strains, which suggested the potential of using differences in restriction sites, and thus insertions/deletions (indels) in nucleotide sequence to discriminate between the other *M. aeruginosa* strains, as well as using the *mcyB* gene sequence (e.g., primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M), to discern between *M. aeruginosa* and *M. wesenbergii* in raw water samples (Fig. 2.1). The presence of the

gene *mcyB* in three of the four environmental strains was indicative of the strains' potential to produce microcystin (Oberholster 2004).

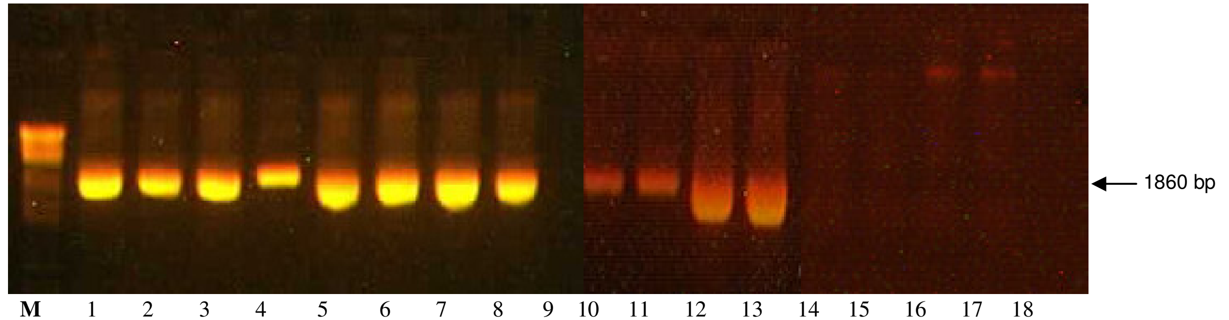

Figure 2.1 PCR fragments obtained after amplification of *Microcystis* strains with primer pairs specific for *mcyB*. Lanes 1-13 represents *Microcystis aeruginosa* strains, and lanes 14-17 represents *Microcystis wesenbergii*. M = Marker III (lambda DNA restricted with *Eco*RI and *Hind*III), 1 = PCC7813 (0.2 µl DNA); 2 = SAG1 (0.2 µl DNA); 3 = CCAP1450/1 (0.2 µl DNA), 4 = UV027 (0.2 µl DNA), 5 = PCC7813 (0.4 µl DNA); 6 = PCC7813 (0.5 µl DNA); 7 = SAG1 (1 µl DNA), 8 = SAG1 (0.8 µl DNA); 9 = water control; 10 = UP01 (0.2 µl DNA); 11 = UP03 (0.2 µl DNA); 12 = UP03 (1 µl DNA); 13 = UP04 (1 µl DNA); 14 = UP02 (0.2 µl); 15 = UP02 (0.2 µl); 16 = UP02 (1 µl); 17 = UP02 (1 µl); 18 = water control (Oberholster 2004).

A vast number of restriction sites were identified with differences followed by restriction digest of the specific polymerase chain reaction (PCR) *mcyB* gene fragment (Oberholster 2004, Figure 2.2; Grobbelaar *et al.* 2004). These reports demonstrate that PCR assays provide a useful indicator of toxicity, as well as the identification of taxonomical characteristics between laboratory cultures and environmental isolates.

2.7 Raw water treatment processes

Traditionally raw water treatment aimed to reduce the concentration of organic matter in drinking water. However, primary and secondary treatments cannot meet anymore the increasingly stringent requirements of water pollution laws and the pressures due to deteriorating raw water quality. Pietsch *et al.* (2002) reported that flocculation and

filtration resulted in an increase of extracellar toxin after experiments with *Microcystis aeruginosa* and *Planktothrix rubescens*. The researchers suggested turbulences in pipes and pressure gradients in the filter as reasons for the increase of the toxin level. The efficacy of chlorine (0.5 mg/L) to eliminate microcystin is also doubtful (Hitzfeld *et al*. 2000). Water treatment studies conducted at the laboratory and pilot plant-scale have concluded that granular activated carbon filtration is effective in removing the cyanobacterial toxins from water (Newcombe *et al*. 2001). This treatment add considerably to the expenses of water treatment and only a few purification water treatment plants in the world is equipped with granular activated carbon systems, the rest make use of conventional water treatment practices that remove live cyanobacterial cells and debris but not biotoxins in solution. In rural areas the choice of water supply may be limited, depending on the stage of development of the country. Similarly, in urban areas if the reticulated drinking water is of doubtful quality, the only choice may be bottled water, which is financially out of reach for the poorer majority of the population. Thus, the potential for injury from biotoxins in water supplies will to some extent depend on the level of development of the country and to some extent on the socio-economic status of the family (Falconer 1999).

Survey analysis of utility waters in the United States and Canada were confirmed to contain microcystin during the sampling period of June 1996 to January 1997. Of the 677 samples collected, 539 (80 %) were positive for microcystin when tested using ELISA. Of the positive samples, 4.3 percent were higher than the WHO drinking water guideline levels of 1µg/L. Only two of the plant outlet samples submitted exceeded the 1-µg/L WHO drinking water guideline. This indicates that, although almost all water treatment plants had adequate procedure to reduce microcystin to safe

levels in the finished water during the test period, the majority of source waters with cyanobacteria do contain microcystin (Carmichael 2001).

2.8 Conclusion

By reviewing the existing information on (i) the impact of anthropogenic activity on water resources, which result in the presence of toxic compounds in water resources, and thus deterioration of water resource quality in general and specific to the US; (ii) toxic chemical pollutants and cyanobacterial toxins and the risk thereof to human health; and (iii) detection methods, my goal was to introduce to the water management professionals the risks involve in poor management strategies. It is important to note that it is not merely high dosage exposures to toxic compounds, but also long-term intakes and exposures that have major detrimental health effects, thus making monitoring and early detection of toxic compounds a high priority. By using biological organisms (i.e., benthic phytoplankton, macroinvertebrate, fish, etc.) as bioindicators of aqautic ecosystem health, an inexpensive tool for assessment of the presence of chemical and biological toxic compounds is provided. For specific biotoxins (i.e., microcystins) the *mcy* gene cluster in PCR assays, applied directly to environmental samples, provide a useful indicator that mixed-species phytoplankton samples may have the genetic potential to produce microcystin. Although HPLC provides a direct measure of toxins present, it does require a large capital investment and considerable sample preparation. The PCR-based assays detect toxigenic cells rather than toxins and require little sample preparation and modest capital costs. Detection of toxic *Microcystis aeruginosa* strains through molecular markers for microcystin may have great use-potential in routine analysis of aquatic ecosystems.

Thus, it may make water monitoring more feasible and allow the early application of corrective action before cyanobacteria blooms start to die or disintegrate. The PCR-based assay is effective at a level of 10 cells/mL and can indicate a possible toxic bloom well before the cell count reaches the action alert at a cell density of 2 000 /mL, as recommended by the Australian Drinking Water Guideline (National Health and Medical Research Council/Agriculture and Resource Management Council of Australia and New Zealand 1996) and a high alert level of 20 000 cell/mL, where blooms may contain sufficient toxin to be of concern for human health (Fitzgerald *et al*. 1999).

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CHAPTER 3

Using a battery of bioassays, benthic phytoplankton and the AUSRIVAS method to monitor long-term coal tar contaminated sediment in the Cache la Poudre River, Colorado.

The contents of this chapter have been published online in *Water Research* 10.1016/j/waters.2005.08.029 (see http://authors.elsevier.com/sd/article/S0043135405004926).

Abstract

This survey provides information on sediment toxicity and structural characteristics of the macroinvertebrates and benthic phytoplankton at 10 locations in the Cache la Poudre River after long-term exposure to coal tar residue. The application of the Australian river bioassessment system (AUSRIVAS) as well as a biotest battery was conducted to evaluate the river 'health' condition. Coal tar is a dense nonaqueous phase liquid of significant environmental concern due to its toxicity and persistence in the subsurface. Organisms like *Selenastrum capricornutum, Daphnia magna* and *Chironomus tentans*, representing different complexities in the biosphere, were selected as test systems for ecotoxicological studies. The results obtained in this study indicate that a biotest battery, macroinvertebrate and benthic phytoplankton communities are in principle, suitable biological tools for evaluation of toxic oil and coal-derived substances in long-term contaminated river sediment.

***Keywords**:* Coal tar, sediment toxicity, biotest battery, river bioassessment

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3.1 Introduction

High levels of contaminants in fresh water sediment may irreversible adverse effects to organisms that inhabit contaminated regions of ecosystems. The exposure to contaminated sediment particles is not only important for benthic organisms (Harkey *et al*. 1994) but also for algae, daphnids, or fish in case of resuspension of the particles (Knezovich *et al*. 1987). Sediment is an integral component of aquatic ecosystems, providing habitat, feeding and spawning for many aquatic organisms. Because

sediment serves as a reservoir for contaminants, it is a source of contaminants to the water column and organisms (U.S. EPA 1997).

Various substances from conventional coal processing, e.g. polycyclic aromatic hydrocarbons (PAH), phenols and cyanides cause strong environmental concern because of their toxic, mutagenic or cancerogenic properties. Coal and oil gasification was a large industry in the United States that operated from the 1800s to the early 1950s. Manufactured gas plants in the U.S. used coal and oil to derive light-end hydrocarbons that were utilized for lighting and heating (Environmental Research & Technology 1984). A major byproduct of gas manufacturing processes was coal tar, a dark-colored, dense nonaqueous phase liquid. Coal tar typically was disposed onsite until abundant natural gas resources have eliminated the need of manufactured gas plants.

Freshwater pollutants have been monitored mainly by physical and chemical techniques (Hattingh 1979). However, these approaches are often impractical and have been deemed inadequate to provide information on unknown hazardous compounds and their potential harmful effects on man and aquatic ecosystems (Cairns & Gruber 1979). Since living organisms will show some response to hazardous levels of any chemicals, the use of biological sensors has become an alternative and increasingly important approach in the prediction and control of water pollution (Cairns *et al.* 1977). In the present study a battery of bioassays was used as a screening tool due to the fact that no single species is sensitive to all chemical effluents in an ecotoxicological risk assessment (Toussaint *et al.* 1995). Multispecies tests that incorporate functional relationships between trophic groups, such as grazers

and producers, can warn of environmental damage resulting from secondary effects that are unpredictable from single species tests alone (Crossland 1984). A desirable property for a test battery member is that its sensitivity pattern should be different from other tests; i.e., to complement each other (Wangberg *et al.* 1995) and therefore both grazers and producers are included in most microcosm toxicity test methods (Cairns *et al.* 1986; Taub *et al.* 1985). Algae and aquatic invertebrates are attractive organisms because their generation spans are shorter than those of higher organisms such as fish.

In this article three different species representing different trophic levels were use to analyze the toxicity of remnants of coal tar in contaminated sediment of the Cache la Poudre River. The selected species were the fresh water algae *Selenastrum capricomutum,* and the invertebrates *Daphnia magna* and *Chironomus tentans*. The aim of the investigation carried out was firstly to determine the ecotoxicological effects of coal-relevant substances and bioconversion products on benthic invertebrates and phytoplankton community in a oil sheen area of the Cache la Poudre River, Colorado, and secondly to use a validated battery of sensitive bioassay to identify highly sediment polluted areas.

3.2 Material and methods

3.2.1. Study area

The Cache la Poudre River originates in the mountains of Colorado near the Continental Divide about 68 km west of Fort Collins. It has a drainage area of about 738 km^2, and flows northeastward about 194 km from its origin to its confluence with

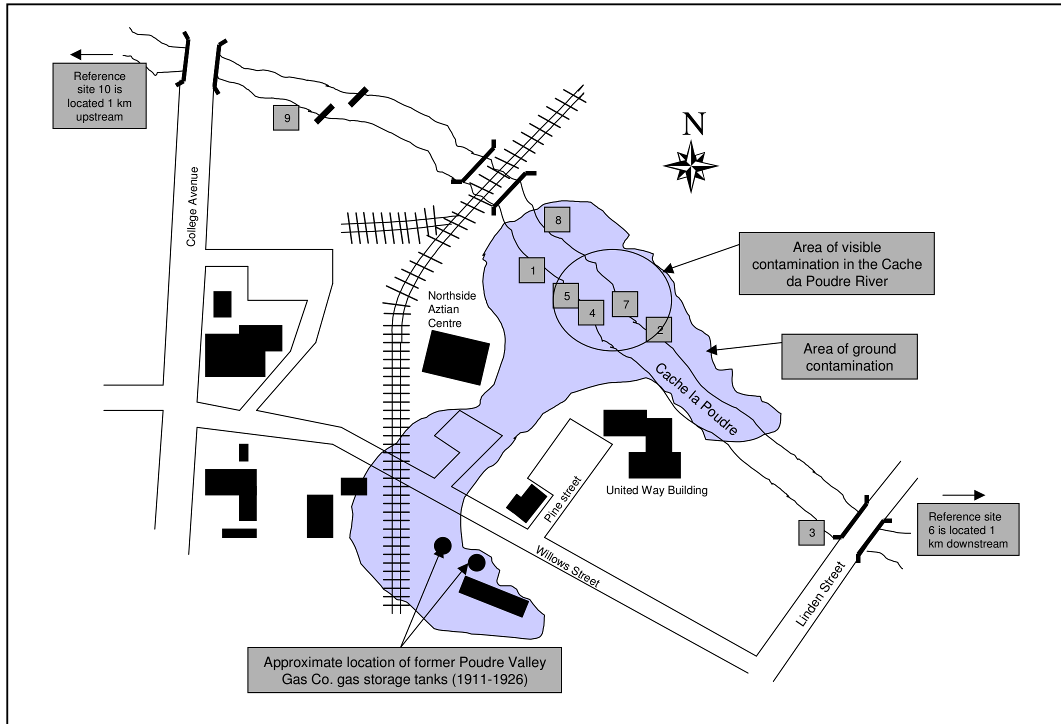

Figure 3.1 Map of the study area in Fort Collins showing the sampling sites on the banks of the Cache la Poudre River, Colorado.

the South Platte River about 8 km east of Greeley. The river basin includes two physiographic provinces, the Southern Rocky Mountain Province and the Great Plains, which correspond to the two major ecoregions, Southern Rockies and Western High Plains (Dennehy *et al*. 1995). The Cache la Poudre River is an important water resource in Ft. Collins, since it supply drinking water to > 200 000 residents in Larimy County. It is further utilized for recreational and irrigation purposes. Water from the Cache la Poudre River also serves as a supplementary water source to the urban lakes (e.g., Sheldon Lake) in Ft. Collins (Sheldon Lake Drainage Improvement Project 2002, 2003a,b; Collins & Sprague 2005). Sampling sites were established on a 150-200 m oily sheen stretch of the Cache la Poudre River east of North College Avenue and west of Linden Street, Fort Collins (Fig. 3.1). The oily sheen was first discovered in September 2002 and is likely the remnants of coal tar, a byproduct from a gas company in business from 1904 to 1926. Over a long period of time, the oily

substance has seeped through the sandy sediment about 4 m beneath the surface and eventually dipped down into the cracked layers of bedrock, spreading into the river (Lingle 2004).

Stream velocity, substratum type (i.e., percentage of cobbles, pebbles, gravel, sand and silt, and embeddedness of cobbles), substratum cover (i.e., filamentous algae, macrophytes and coarse particulate organic matter), and canopy cover were determined at each location where the phytobenthos was sampled. Substratum type and substratum cover were determined visually (Stevenson & Bahls 1999).

3.2.2 Toxicity testing

3.2.2.1 *Selenastrum capricornutum* biotest

In this study we used a 96-hour growth, standard freshwater algal toxicity test with the unicellular, crescent-shaped, green alga *Selenastrum capricornutum* (40-60 μm^3) that can be found in both eutrophic and oligotrophic freshwater environments. Methods used for culture and testing were as described by the Environmental Protection Agency (EPA)(U.S. EPA 1994). This method was originally developed for use with effluents, receiving waters, and pure compounds, but it is also suitable for testing sediment. The test was used to measure changes in cell density and chlorophyll content. Starter algae cultures were obtained from Aquatic Bio Systems Inc. Fort Collins, Colorado. Instructions for the preparation of glassware and nutrient stock solution were as detailed by the EPA (U.S. EPA 1994). Cultures were grown in a

nutrient medium that include EDTA and then tested in a medium without EDTA. The cultures were grown in 250-mL glass Erlenmeyer flasks containing 100 mL culture medium. New cultures were started each week. The flasks were sealed with foam plugs to prevent contamination and placed in an incubation chamber at 25 ± 1 °C under continuous illumination (60-80 μE/m^2/s) supplied by cool light fluorescent lamps. The toxicity test was initiated after the stock cultures have reached a cell density of 1 x 10^2 cells/mL and were entering a log-phase growth stage. The same amount of sediment pore water obtained at the different sampling sites, while sampling macroinvertebrates with a core sampler, was filtered through a 0.45 μm filter to remove particle material. The test was conducted as a screen (100 % concentration). Nutrients without EDTA were added to ensure that reduction in growth was not due to nutrient limitation. The toxicity test was conducted in 250-mL Erlenmeyer flasks, with a test volume of 100 mL and three replicates per treatment. The negative control consisted of three replicates of the culture medium, prepared without EDTA. The inoculum density was adjusted to 1 x 10^6 cells/mL and each flask was inoculated with 1 mL stock culture. Each of the flasks were sealed with a foam plug and placed in an incubation chamber at 25 ± 1 °C with a continuous photoperiod. The flasks were swirled twice daily by hand and randomly repositioned in the incubation chamber. Cell density and chlorophyll*a* (Chl*a*) contents were measured of the triplicate samples at 24, 48, 72 and 96 hours and the mean count (cells/mL) was calculated for each treatment. Chl*a* was extracted from lyophilized GF filters using N,N-dimethylformamide for 2 h at room temperature. Chl*a* was measured photospectrometrically at 647 and 664 nm and calculated according to Porra *et al.* (1989). The following water quality measures: pH, alkalinity, hardness and temperature were taken at test initiation and termination.

3.2.2.2 *Daphnia magna* test

Standardized techniques involving whole sediment exposures, with acute or sublethal endpoints, have been used in the 48-hour toxicity test (American Society for testing and Materials, ASTM 2000). Test organisms *Daphnia magna* < 24 hour neonates were obtained from Aquatic Bio Systems Inc. Fort Collins, Colorado. They were cultured at 22 °C, under a photoperiod of 16:8 h light:darkness and a light intensity of 10 Ue/m^2/s. The culture medium was deionized water, with a pH 7.99, total hardness of 250 mg $CaCO_3$/L and total alkalinity of 96 mg $CaCO_3$/L. A static 48-hour acute test, with whole sediment samples, was conducted as a screen (100 % concentration). Each test container was a 250-mL glass beaker, and there were five replicates per treatment. Approximately 50 g sediment (wet weight) was placed in each beaker, and then 200 mL culture water was added, taking care not to disturb the sediments. After a settling period of 3 days as described (ASTM 2000), ten neonates were added to each container, and care was taken to release them below the water surface. For the negative control, the *Daphnia* were kept in pure water. Dissolved Oxygen (DO) and temperature was measured daily, while pH, ammonia, conductivity, alkalinity, and hardness were determined at test initiation and termination. After 24 h and 48 h the number of immobilized *Daphnia* was determined visually. *Daphnia* that sank to the bottom of the vessels within 15 s of having been slightly shaken were considered immobile. Data from the replicate beakers were pooled and treatments were compared to the negative control and reference sites.

3.2.2.3. *Chironomus tentans* test

The assessment of whole sediment toxicity involves a 10-day exposure of *Chironomus tentans* to the contaminated sediment. We conducted the toxicity test in 300-mL beakers with 8 replicate chambers. The sediment volume for the test was 100 mL with 175 mL of overlying water. Sediment of the different sampling sites were prepared the day before test initiation and allowed to equilibrate overnight following the instructions of the U.S. EPA (2000). The following day 10 second-to third-instars larvae were added per chamber under a 16:8 h light; dark photoperiod at 23 ± 1 °C. Overlying water in the test containers were renewed, with two volume replacements per day (U.S. EPA 2000). Hardness, alkalinity, conductivity, pH and ammonia were monitored at the beginning and end of the test while temperature and DO were monitored daily. After 10 days the mean survival of larvae exposed to test samples were compared to the mean survival of larvae exposed to controls and reference samples.

3.2.2.4 Data analyses of biotest endpoints

The test results were recorded on standard Excel spreadsheats for data processing, and statistical analysis was performed using SYSTAT® 7.0.1 (1997). The responses of bioassay samples were subjected to an analysis of variance ($p \leq 0.05$) using SYSTAT® 7.0.1 (1997) compared to examine the endpoint variability of each test. All toxic responses were modeled by probit function (LT50) where number of dead/total number is a probit function of time.

3.2.2.5 Sampling of macroinvertebrates and phytobentos

Macroinvertebrates and phytobentos were collected at weekly intervals with a corer at each of the ten sampling sites from August to October 2004. Four core samples were taken monthly at the ten sampling sites (Fig. 3.1). A random sampling procedure was used to reduce hydrobiological variability between sites (Voelz & Ward 1991). The corer was driven into the substrate to an approximate depth of 10 cm. Cobble-sized rock within the corer were transferred to a bucket and scrubbed. The other material within the corer was removed by hand. Macroinvertebrates, organic matter, and remaining water were passed through a sieve with 75 μm apertures. These materials were placed in labeled 0.5-L glass jars with plastic lids and preserved in 10% (v/v) formalin solution. The sediment water was fixed with buffered 5% (v/v) formaldehyde for determination of benthic phytoplankton composition, community structure and identification of taxa. Cells greater than 30 μm in diameter were counted first at x 125 magnification; smaller taxa were then enumerated at x 1250 using the strip-count method American Public Health Association (APHA 1989). Diatoms were identified after clearing in acid persulphate. Biovolumes of more abundant taxa were estimated by measuring cell dimensions of at least 20 individuals and using closest geometric formulae (Willen 1976). Identification of taxa was done according to described methods of Krammer and Lange-Bertalot (1986-1991) and Patrick and Reimer (1975). In the laboratory, the benthic samples were washed through a series of three sieves to separate the following size classes: > as 1 mm, 250 μm-1 mm, and 75-250 μm. Each sample was sorted with an illuminated dissecting microscope at 20x magnification. Organisms were counted and placed in labeled glass vials containing 70% (v/v) ethanol before taxonomic identification to the lowest possible taxonomic

category, according to Merritt and Cummins (1996) and Thorp and Covich (1991). The macroinvertebrate and benthic phytoplankton collected at the reference sites were analyzed and used to build an Australian River Assessment System (AUSRIVAS)-type predictive model (Table 3.1) for the oil sheen area.

Table 3.1 O/E index and ecological health rating for the long-term contaminated sediment-sampling sites in the Cache la Poudre River.

Site	O/E*	Rating
1	0.43	Moderately impacted
2	0.00	Severely degraded
3	0.44	Moderately impacted
4	0.00	Severely degraded
5	0.00	Severely degraded
6	0.68	Mildly impacted
7	0.00	Severely degraded
8	0.45	Moderately impacted

*E number of taxa expected from those predicted with > 50 percent probability of occurrence; 0, number of taxa observed from the list of those with > 50 percent probability of being at the site; O/E, Observed/Expected ratio. This should be near 1.0 for sites similar to reference conditions in the Cache la Poudre River. Sites with O/E < as 0.8 are below reference in the Cache la Poudre River.

The habitat data of the eight sites judged to be damaged were compared with the reference sites to provide an assessment of the ecological health of the river in damaged areas (Simpson & Norris, 2000; Humphrey *et al.* 1995, 2000). Only those taxa which had a probability of 50% or greater of occurring at a damaged sampling site, were used to calculate the number of expected (E) taxa. The observed (O) number of taxa was obtained by summing the number of taxa actually recorded at the damaged sampling site with expected probabilities of > 50 percent. The O/E index was calculated to provide a measure of the difference between the observed and expected macroinvertebrate and benthic phytoplankton distributions. An O/E ratio

near 1 is equivalent to reference while a ratio less than about 0.8 indicates that 20% of the expected taxa were missing in the benthic environment of the damaged site and it is below reference condition.

3.3 Results

3.3.1 Physicochemical characteristics

The substratum at each sampling site in the Cache la Poudre River consisted predominantly of pebbles, sand and silt-clay. These three size fractions collectively constituted over 85% of the mineral substratum, while cobble stones were only present at sites 1, 8, 9, 10 (Fig. 3.2).

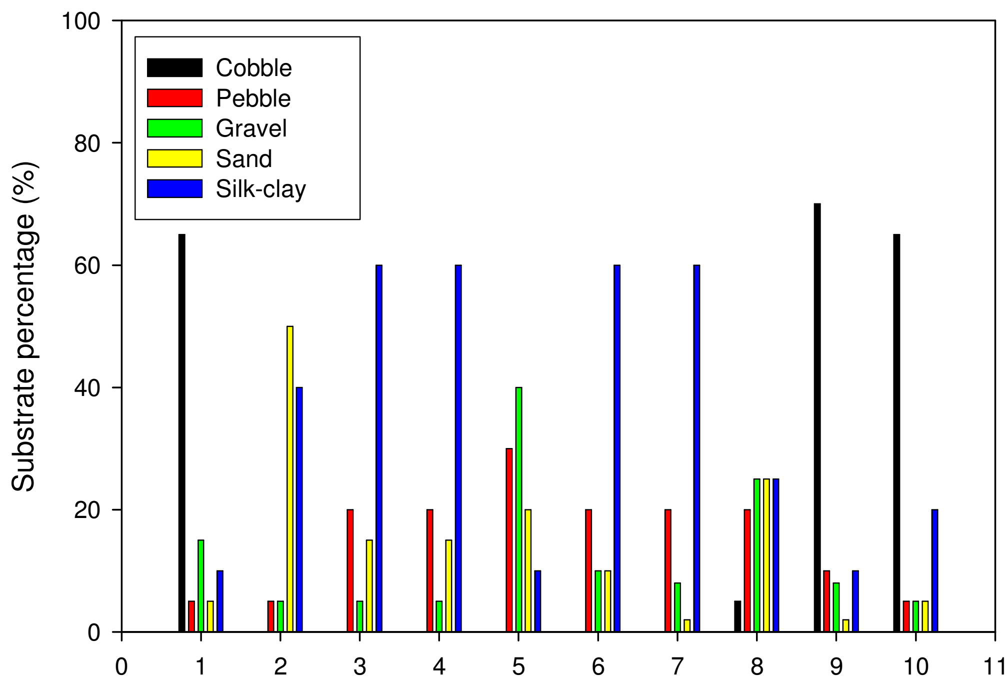

Figure 3.2 Substratum composition of the ten sampling sites in the Cache la Poudre River, Colorado**.**

The flow regime was measured on six occasions when the flow was high, medium and low, respectively (Fig. 3.3). During the whole period of our investigation the pH at all of the ten sampling sites was above seven (Fig. 3.3).

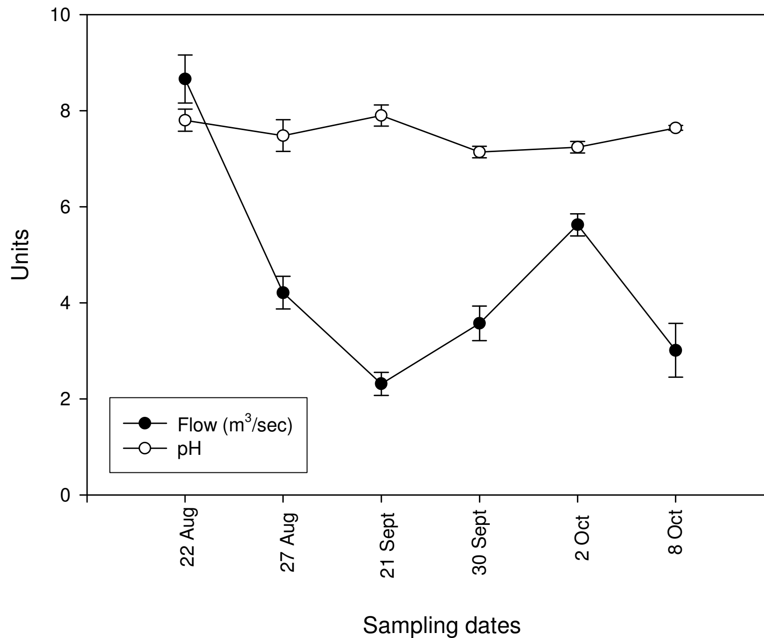

Figure 3.3 Variations in flow and pH from August 22 to October 8 in the Cache la Poudre River, Colorado. Error bars indicate standard deviations from the mean value.

3.3.2 *Selenastrum capricornutum* biotest

Results of the present study indicate that the values of Chl*a* content were subjected to considerable variation according to the different sampling sites and the exposure period (Fig. 3.4b). The control and reference sites 9 and 10 revealed no significant differences in Chl*a* content upstream. In the case of sampling sites 2, 4, 5 and 7, a

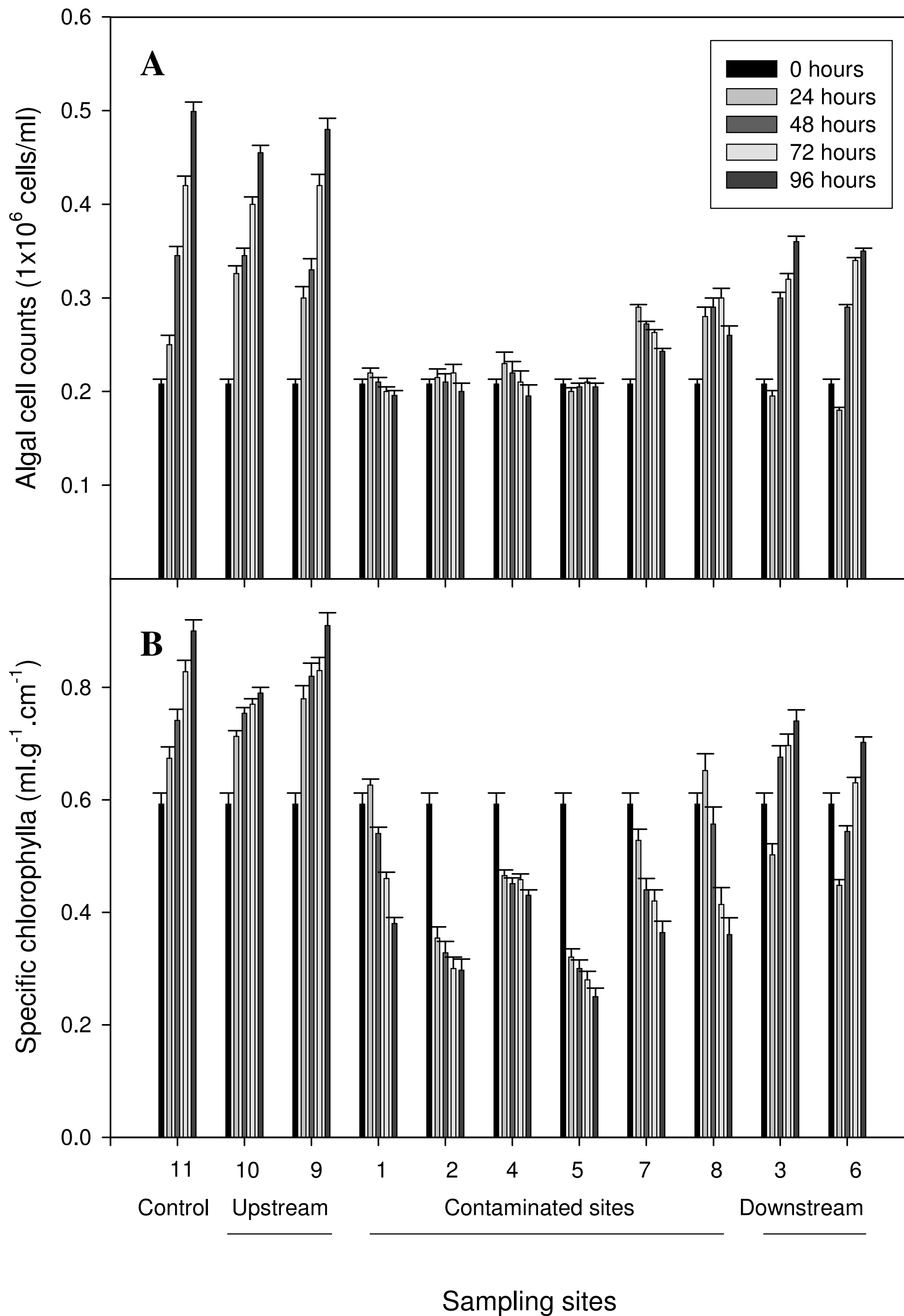

Figure 3.4 Changes in *Selenastrum capricornutum* cell counts (A) and specific Chl*a* content (B) at the different sampling sites in the Cache la Poudre River, Colorado. Error bars indicate standard deviations from the mean value.

decrease in Chl*a* content of *Selenastrum capricornutum* was significant during the whole exposure time of the experimental run. On the other hand, at sampling site 3 and 6 downstream, the *Selenastrum capricornutum* growth was inhibited up to the third day of incubation and thereafter, the *Selenastrum capricornutum* started to recover. Variation in counts of *Selenastrum capricornutum* cells of the different sampling sites over a period of 96 hours is represented in Fig. 3.4a. At sampling sites 3 and 6, *Selenastrum capricornutum* started to recover by the end of the third day of exposure and attained a higher count at the end of the 96 hours while sampling sites 2, 4, 5 and 7 resulted in a decreased of *Selenastrum capricornutum* cell count by the end of the exposure time. The total *Selenastrum capricornutum* count decreased in response to the coal tar residue compared with the control and reference site 9 and 10.

3.3.3 Fresh water *Daphnia magna* and *Chironomus tentans* biotests

The percentage of survival of daphnids in 100% concentration for 48 hr of exposure is indicated in Fig. 3.5. At sampling sites 2 and 5, the survival of *Daphnia magna* to the whole-sediment toxicity test was 0% after 48 h of exposure, while at sampling site 7 only 20% survived. Coal tar residue had little effect on the survival rate of *Daphnia magna* at sampling sites 3 and 6 downstream.

The survival endpoint results for *Chironomus tentans* following a 10-day exposure to whole-sediment toxicity test are illustrated in Fig. 3.5. The sediment of sites 9 and 10 were used as a reference. Sampling site 5 was highly toxic and contained 13.3% survivors, while sampling sites 2, 4 and 7 had a survivor percentages of 40, 40,

26.66%, respectively. The trend observed in this data set is comparable to the data obtained from the *Selenastrum capricornutum* and *Daphnia* bioassay, and is highly significant ($p \leq 0.001$).

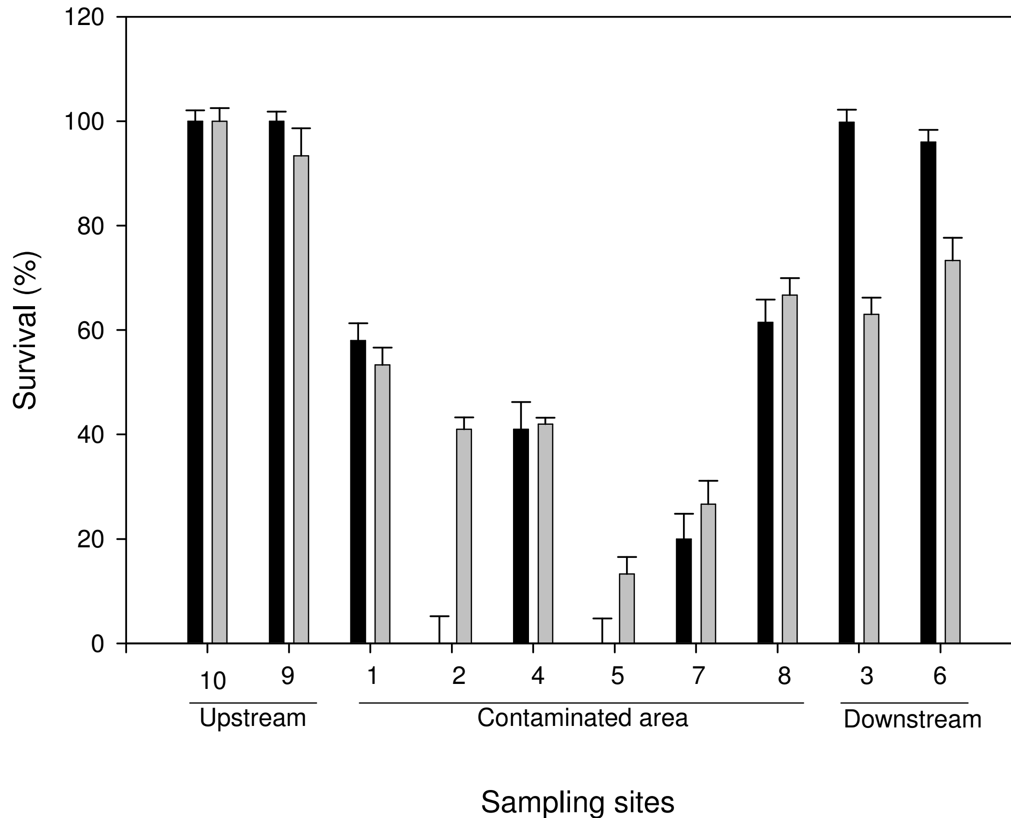

Figure 3.5 Results of the survival rates of *Daphnia magna* (48 h) and *Chironomus tentans* (240 h) after biotesting of sampling sites. Error bars indicate standard deviations from the mean value.

3.3.4 Macroinvertebrate Response

Eighty percent of the organisms collected at the sampling sites belong to the following taxonomic groups: Annelida (Oligochaeta), Diptera (Chironomidae), Ephemeroptera

(*Tricorythodes minutus*), Tricoptera (*Hydropsyche* spp.) and Nematoda, while the relative abundance of other taxonomic groups was low (not shown). The highest diversity and evenness were both recorded at site 10, while the habitat at the reference and most other sampling sites did not support any aquatic vascular plant biomass. Analysis of data upstream and downstream of the oily sheen revealed that there were a significantly higher number of organisms with a low number of taxa downstream at sites 3 and 6, than upstream ($p \leq 0.0029$). Only a few taxa occurred at these sites, and diversity values at locations 3 and 6 were consistently lower than the reference locations 9 and 10 upstream during the sample period. The major taxa, comprising of collector-gatherers (Chironomidae; Oligochaeta) was much higher at sites 9 and 10, upstream from the oily sheen, than at the other sampling sites. Sites 3 and 6 downstream had the highest relative abundance of collector-gatherers (Nematoda, Oligochaeta), followed by lower numbers of collector-filterers (Trichoptera) during our survey (Fig. 3.6). Very few chironomids, scrapers and shredders were either eliminated or reduced in numbers at the sampling sites.

Using the AUSRIVAS-method, it was found that sampling sites 2, 4, 5 and 7 were severely degraded with none of the expected macroinvertebrate families found. The sampling sites 9 and 10, which were ± 200 m and 1 km upstream, respectively from the oily sheen were selected as unmodified reference sites and used as a baseline for judging change.

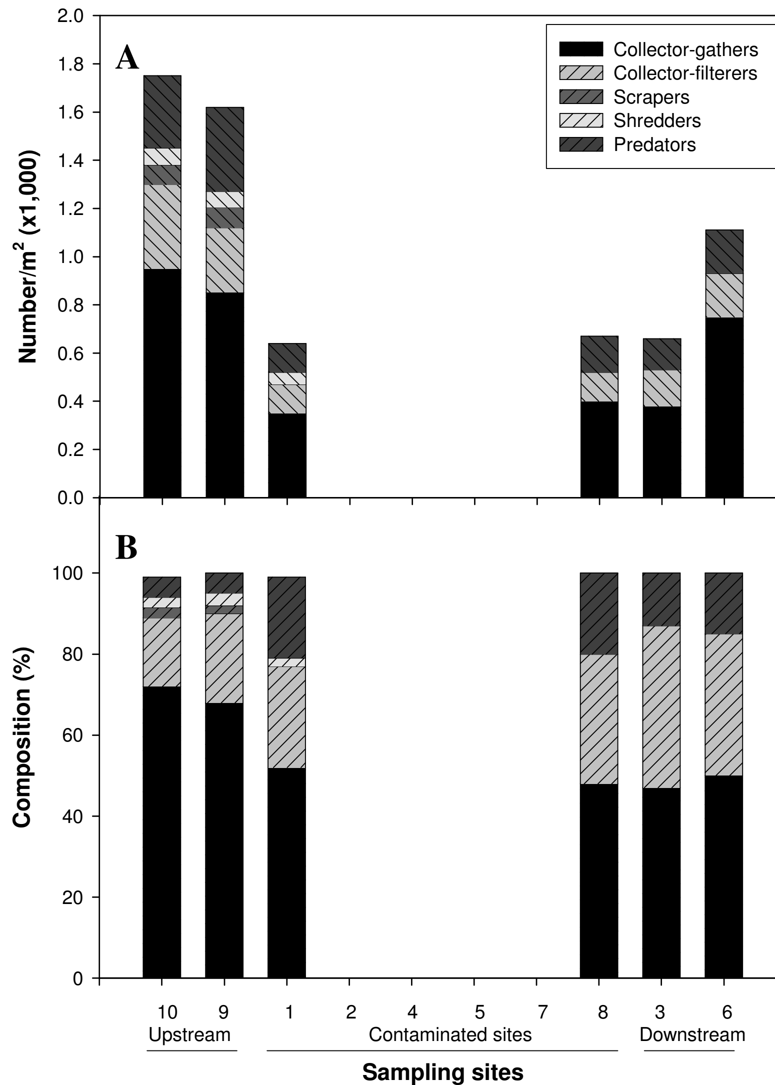

Figure 3.6 (A) Abundance (individuals/m^2) and (B) percentage composition of macroinvertebrate functional feeding groups recorded at the 10 sampling sites on the Cache la Poudre River, Colorado. Mean values from sampling during August to October, 2004.

3.3.5 Benthic phytoplankton response

High diversity of species and abundance were recorded in case of diatoms with respect to the two reference sites 9 and 10. Green algal species were relatively

uncommon at all the sampling sites. The only genera that dominate the contaminated areas was *Didymosphenia geminata* (Fig. 3.7).

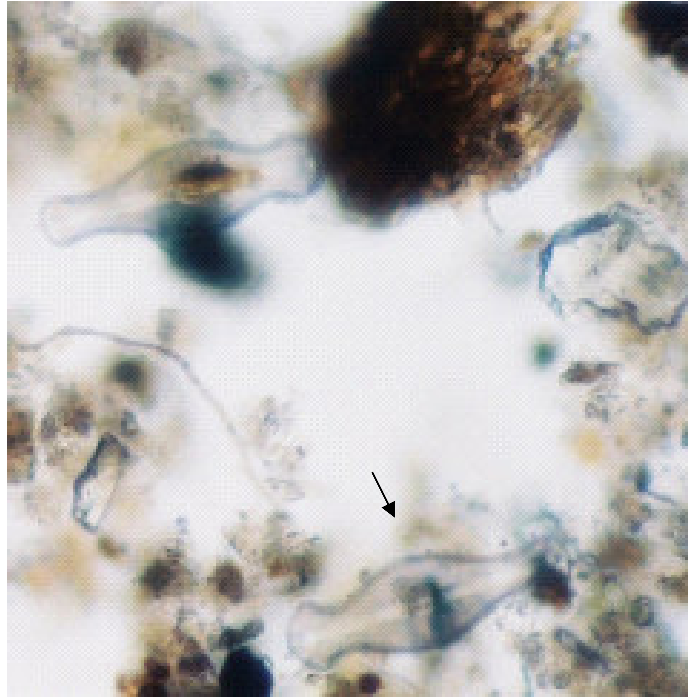

Figure 3.7 Bacillariophyceae, *Didymosphenia geminata* (indicated by a arrow), the only taxon in large abundance in the contaminated sediment. Unstained, bright-field microscopy, 200 x.

This diatom genera was much more abundant in the highly contaminated sampling sites 2, 4, 5, and 7 than at any other site upstream or downstream, including the reference sites 9 and 10 (Fig. 3.8).

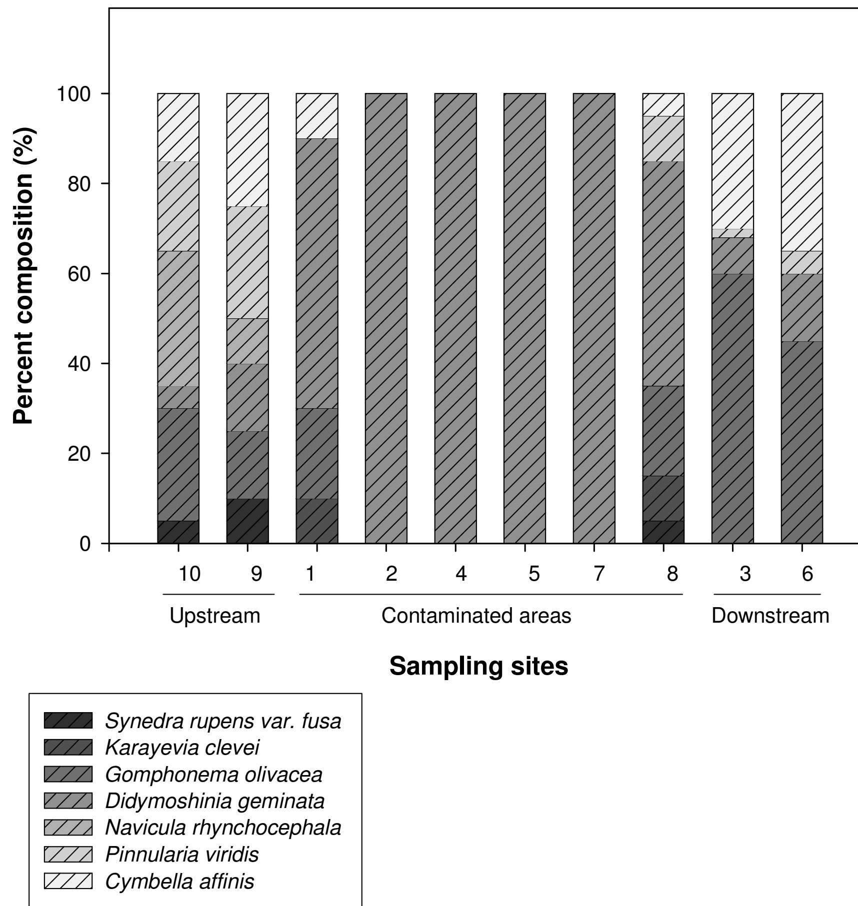

Figure 3.8 Percentage composition of different Bentic phytoplankton species recorded at the 10 sampling sites on the Cache la Poudre River, Colorado. Mean values from sampling during August to October, 2004.

Comparisons with the reference sites 9 and 10, revealed that sampling sites 3 and 6 downstream from the oily sheen, have a lower species diversity than the upstream sites, and that only a few taxa occurred at these sites of which the genera *Gomphonema olivacea* and *Cymbella affinis* were the major taxa with *Didymosphenia geminata* in much lower abundance.

3.4 Discussion

3.4.1 Physicochemical characteristics

Douglas (1958) and Cattaneo *et al*. (1997) recognized that different sizes of stone supported different densities and species of epilithic algae, which was likely the result of differences in their susceptibility to flood disturbance. Distribution of stream macroinvertebrates is also closely related to substrate characteristics (Minshall 1984). The substratum in the Cache la Poudre River consisted predominantly of pebbles, sand and silt-clay (Fig. 3.2). The flow regime was measured (Fig. 3.3), since this is an important factor shaping structure of benthic-algal assemblages in the Cache la Poudre River due to the fact that diatom immigration onto bare substrata may increase with reduced current speed (Stevenson 1983). The influence of velocity, like discharge, may interact with other variables (e.g., substrate, food supply, DO) to determine habitat conditions for macroinvertebrates (Ward 1992). Winter *et al*. (2003) reported that a higher proportion of diatom species were indicative of a pH > 7 and a lower proportion of species indicative of a pH < 7 which showed that the structure of benthic-algal assemblages correlated strongly with pH.

3.4.2 *Selenastrum capricornutum* biotest

Phytoplankton, benthic and epiphytic microalgae, and macroalgae are energy sources critical to most aquatic ecosystems. Changes in their density and composition can affect the chemical and biological quality of the habitat (Round 1981). The impacts of petroleum pollutants on algae are subject to variation due to the great variability in

both the chemical composition of the pollutants, as well as algal population (Morales-Loo & Goutz 1990; Herman *et al*. 1991). Furthermore, algae have been shown to be more sensitive to complex wastes than fish or invertebrates (Miller *et al*. 1978). Results of the present study indicate that the values of Chl*a* content were subjected to considerable variation according to the different sampling sites and the exposure period (Fig. 3.4b). The total *Selenastrum capricornutum* count decreased in response to the coal tar residue compared with the control and reference sites 9 and 10. This findings support previous studies by Amman and Terry (1985) and Tukaj (1978) with respect to the effect of several organic pollutants on algae.

3.4.3 Fresh water *Daphnia magna* and *Chironomus tentans* biotests

The water flea *Daphnia magna*, is the recommended and perhaps, the most widely used test organism for toxicity. Tremendous numbers of papers are available regarding the use of daphnids in such evaluation, through the estimation of the median lethal concentration (LC50) or Time (LT50) values (ISO 1982). Fig. 3.5 shows the percentage of survival of daphnids in 100% concentration for 48 hours of exposure. The response of *Daphnia magna* to the whole-sediment toxicity test was similar to the *Chironomus tentans* and *Selenastrum capricornutum* bioassay test, excepted for sampling sites 3 and 6 downstream which correlated strongly with the reference sites 9 and 10. This indicates that *Daphnia* was not as sensitive to exposure of coal tar residue downstream as in the case of the *Selenastrum capricornutum* and *Chironomus tentans* bioassays.

The accumulation of pollutants is maximum in the upper few centimeters of sediment which are important for a number of biological processes (Levin & Kimball 1985). *Chironomus tentans* is therefore an ideal species for toxic sediment assessment because it comes into contact with the compounds through both physical contact and ingestion of sediment material. Furthermore researchers also have demonstrated that natural factors do not significantly affect the survival, growth, and reproduction of *Chironomus* ssp. (Ankley *et al*. 1994; Day *et al*. 1995b).

3.4.4 Macroinvertebrate Response

Macroinvertebrate communities have been commonly used to assess stream water quality, and most studies on environmental monitoring and assessment of stream ecosystems focused on the change of community structure or functional organization of macroinvertebrate as responses to environmental stresses (Rosenberg & Resh 1993).

The organisms in the major taxonomic groups: Annelida (Oligochaeta), Diptera (Chironomidae), Ephemeroptera (*Tricorythodes minutus*), Tricoptera (*Hydropsyche* spp.) and Nematoda accounted for more than 80% of the organisms collected at the 10 sampling sites. However, the relative abundance of other taxonomic groups was low, which may be partly due to the absence of any acquatic plant biomass. Gregg and Rose (1985) indicated that aquatic plants increased physical heterogeneity and created more diverse habitats for the attachment of benthic macoinvertebrates. Cummins (1974, 1975) suggested that food resources are a major determinant of stream macroinvertebrate distribution and proposed a classification of stream

macroinvertebrates into functional feeding groups (Cummins 1974). Distributional patterns of functional feeding groups reflect resource distribution and use, and facilitate the understanding of organic matter processing in river ecosystems (Vannote *et al*. 1980). Therefore, it has been suggested that the bioassessment of river water quality based on functional feeding groups of macroinvertebrates may be superior to that based on community structure alone because it reflects more ecologically significant attributes of rivers (Rabeni *et al*. 1985). In our study the mean number of taxa in each functional feeding group significantly differed among the 10 sampling sites. The major taxa, comprising of collector-gatherers (Chironomidae; Oligochaeta) was much higher at the reference sites, upstream from the oily sheen (Fig. 3.6). Few scrapers and shredders were observed downstream that probably indicates the effect of oily substances in the water. Other studies have observed higher abundances of some chironomid and oligochaete taxa related to petroleum contamination (Rosenberg & Wiens 1976; Woodward & Riley 1983), however no apparent oil-induced increases in these groups were observed downstream of the oily sheen during our study. The most severely affected taxa in the study were Chironomidae, Trichoptera and Ephemeroptera; the sensitivity of these groups to petroleum exposure has been documented elsewhere (Barton & Wallace 1979).

The AUSRIVAS-method, widely used in the USA and Canada (Reynoldson & Metcalfe-Smith 1993), was used in the survey study of the Cache la Poudre River (Table 3.1). Sampling sites 2, 4, 5 and 7 were severely degraded with none of the expected macro-invertebrate families found, which show a good parallelism with the results of the battery of bioassays and also indicated the areas of highly contaminated sediment, because due to the complexities associated with coal tar migration in

subsurface media many coal tar accumulations are difficult to locate (Cohen & Mercer 1993).

3.4.5 Benthic phytoplankton response

Algae are ubiquitous, ecologically important, and sensitive to a broad range of stressors and respond rapidly to changes in water chemistry (McCormick & Cairns 1997). Variation in community structure and function within benthic macrohabitats results from local variation in abiotic forces that are not always easily measured or readily apparent to the unaided eyes. For example, small-scale changes in current velocity (e.g., 5 cm/s) can alter patterns of algal biomass accumulation on the substrate (Stevenson 1983) and therefore sampling was taken at different current velocities (Fig. 3.3). The epipsammic community in the Cache la Poudre River, dominated by diatoms, was very firmly attached to sand particles, and it seems to be well adapted for existence in a highly variable environment (Miller *et al.* 1987). The best use of diversity-related indices in river assessments is probably as an indicator of changes in species composition when comparing impacted and reference assemblages (Jüttner *et al.* 1996). Some investigators have found that diversity decreases with pollution (Rott & Pfister 1988), that diversity can increase with pollution (van Dam 1982), and that diversity changes differently depending upon the type of pollution (Jüttner *et al.* 1996). Patrick (1973) furthermore predicted that some pollutants would differentially stimulate growth of some species and thereby decrease evenness of species abundances, while toxic pollution could increase evenness and that severe pollution could decrease species numbers. In the case of our study high diversity of species and abundance were recorded in case of diatoms with respect to the two

reference sites 9 and 10 (Fig. 3.8). Morales-Loo and Goutz (1990) and Herman *et al*. (1991) found that petroleum hydrocarbons in the aquatic environments could have subtle effects on the relative abundance of algal species, inhibiting the growth of some sensitive species and promoting the growth of tolerant ones.

We found in our study that the use of benthic diatoms as indicators of environmental conditions in the Cache la Poudre River was important for three basic reasons (1) their importance in ecosystems; (2) their utility as indicators of environmental conditions, and (3) their ease of use. Diatom importance in river ecosystems is based on their fundamental role in the food webs (Lamberti 1996), oxygenation of surface waters and linkage in the biogeochemical cycles (Kim *et al*. 1990; Mulholland 1996). As one of the most species-rich components of river communities, diatoms are important elements of biodiversity and genetic resources in rivers (Patrick 1961).

3.5 Conclusion

In conclusion, based on data we suggest that *Selenastrum capricornutum*, *Daphnia magna* and *Chironomus tentans* should be included as a battery of tests for assessing acute toxicity from coal tar, although time, conditions of exposure, and species specificity were different and may have influenced the results. The toxicological properties of coal tar contaminated sediment are currently unknown. However, since neat coal tar has been demonstrated to be highly carcinogenic to laboratory animals, it is reasonable to presume that coal tar contaminated soil represents a concern to human health (Goldstein *et al*. 1998; Gaylor *et al*. 2000). In addition, the presence of coal tar residue contaminated sediment in the environment brings about significant

implications in the community structure of aquatic organism as seen in our study. In the present study we have focused on the exposure of macroinvertebrates and phytoplankton to residue of coal tar and the evaluation of lethal responses of species under laboratory conditions and on the benthic community's response under natural conditions when challenged with a long-term exposure. Because of the numerous routes of exposure we found that, almost any species may be affected by coal tar. Also, because coal tars differ in their chemical composition from site to site based on production methods and temperatures, fuel stock, length and method of storage, leaching of chemicals by nearby soils and water resources, and weathering conditions, the use of coal tar collected from a single manufactured gas plant site as in the case of our study, do not adequately represent the environmental effects of all coal tars. The dominance by a single phytoplankton taxon that was significantly higher at the contaminated sampling sites, than in the case of the reference areas were most probably due to environmental stress of coal tar residue. Nutrient or organic enrichment, or toxic conditions, in streams can cause such shifts in algal communities from dominance by one species to dominance by other taxa (Gausch *et al.* 1998; McCormick & Stevenson, 1998). Such changes in algal taxonomic composition can profoundly affect food web interactions and ecosystem dynamics (McCormick & Cairns 1997).

The results presented in this study show that the importance of the biotest battery in the determination of toxicity of contaminated sediment lies in the fact that these are measures of the organism's response to simultaneous influences of various environmental parameters affecting its toxicity. The main point of the test battery was to include living organisms at two or more trophic levels to determine the potential

effects based on diverse endpoints. Although we did not attempt to measure residue concentrations of the coal tar, we found that the biotest battery is a very useful, sensitive and inexpensive tool to detect toxicants in the environment. The results of our study indicate that changes in the macrovertebrates and benthic algae composition can also be used as bioassessment to identify potential environmental hazards at polluted coal tar sites.

Figure 3.9 Clean-up of the Cache la Poudre, 2004-2005. Before (A) and during (B) restoration.

In October 2004, after reaching an agreement that divided the cost of the cleanup project among Xcel Energy, which operated the gas plant, Schrader Oil Co., which now owns much of the former gas plant property, and the city of Fort Collins, which owns property south of the river contaminated by coal tar and other chemicals, EPA contractors began excavating the contaminated property behind Northside Aztlan Community Center, 200 Willow St. The EPA's plan is to divert and drain a portion of the river, remove as much contaminated soil and groundwater as it could from the riverbed and the contaminated site behind Aztlan, then install a 680 foot long plastic barrier between the contaminated site and the south bank of the Poudre River (Darst 2005) (Fig. 3.9).

Acknowledgements

The authors would like to express their gratitude to the Water Research Commission and the National Foundation of Research of South Africa for their financial assistance.

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CHAPTER 4

Population dynamics and ecological changes in an urban artificaly mixed shallow lake in Colorado, one year after restoration.

The contents of this chapter have been submitted for publication to *Lakes & Reserviors: Research and Management*.
(Manususcript submission number: 2005/16; Date of submission: 22 April 2005)

Abstract

Ecological conditions and phytoplankton succession in Sheldon Lake were analyzed from 2004 to 2005, one year after restoration of this shallow urban lake, in order to determine the trophic state and environmental variables controlling the phytoplankton, macrovertebrates and zooplankton compositions. The anthropogenic eutrophic process of the lake is characterized by increased nutrient concentrations, due to input of urban runoff. This addition triggers a chain of events starting with a massive increase in the growth of primary producers, since these are generally growth-limited by nutrients in freshwater ecosystems. Although the lake was artifically mixed, cyanobacteria were dominant as primary producer during the whole summer until they collapsed and were then replaced by diatoms after a period of high winds and rainfall. The absence of macrophytes in the main basin due to bottom sediment removal had a major effect on the juvenile blue gill sunfish, macroinvertebrates, and *Bosmina* sp. As an alternative *Bosmina* sp. used the surface blooms of cyanobacteria in summer as refuge from grazing by planktivorous fish. The greatest disturbance on the macroinvertebrate community richness and eveness was observed in the areas of incoming stormwater inlets.

Keywords: cyanobacteria, artifical mixing, bottom sediment removel, macrophytes, urban runoff

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4.1 Introduction

Water quality within urban water bodies is often under great pressure, owing to impacts such as storm water nutrient and contaminant loading. Contamination problems in natural receiving waters are complex, generally involving mixtures of chemicals in one or more matrices (e.g., water, sediment and biota). Especially prevalent within urban water bodies are concerns about eutrophication. Many of the same pesticides and nutrients used in large-scale agricultural practices are also used by municipal entities including urban and rural households. Runoff from fertilized lawns, household termite control, city streets, construction sites, storm sewers, household waste, organic nutrients from grass clippings may alter the soil makeup and can render a habitat unsuitable for certain organismes. US municipal discharges affect 16% of rivers and streams, 17% of lakes and reservoirs, and over 35% of estuarie (US EPA 1994).

Skinner *et al*. (1999) showed that stormwater runoff produced significant toxicity in the early life stages of medaka (*Oryzia latipes*) and inland silverside (*Menidia beryllina*). Developmental problems and toxicity are strongly correlated with the total metal content of the runoff and corresponded with exceedences of water quality criteria of Cd, Cu and Zn. The deterioration of water quality manifests itself in changes in the chemical, physical and biological characteristics of the receiving waters. These alterations include the following, reduction in water transparency, increase in pH, deoxygenation of the hypolimnion and the accompanying production of H_2S and CH_4, and release of nitrogen (N), phosphorus (P) and iron (Fe) from sediments during periods of anoxia. The increase in biological productivity which characterizes eutrophication has negative biotic impacts such as (a) an increase in

nuisance phytoplankton species and a reduction in macroalgal and higher plant species due to decrease in water transparency; (b) shifts from plankton-based to benthic or detrital-based food webs due to toxicity, poor palatability and the relative immunity to grazing of bloom phytoplankton species; (c) the toxicity of water contaminated by neuro- and hepatotoxins produced by certain bloom-forming cyanobacteria to resident invertebrates and fish; and (d) interference with recreational use due to water discoloration, and foul odours assocated with blooms (Harper 1992). The objective of this study was to describe the ecological status, as well as the limnological condition of Sheldon Lake a year after restoration based on water quality, phytoplankton, zooplankton and macroinvertebrate community structure. Phytoplankton is the major primary producers in many aquatic ecosystems and as such an essential component of the trophic structure of freshwater. Any changes in phytoplankton community structure or function due to e.g., urban runoff are likely to be reflected at higher levels of the food web.

4.2 Materials and methods

4.2.1 Study area

The study was conducted in Larimer County, Colorado. The 6.07-hectare Sheldon Lake is a shallow man-made lake that was excavated in 1874, and it is a focal point for recreational activities for Fort Collins residents (Fig. 4.1). During December 2002 the Sheldon Lake Drainage Improvement Project was started with the initial purpose of removing over 250 structures from the 100-year floodplain which will provide flood protection by increasing the stormwater detention of Sheldon Lake, especially

after a major flood in 1997 causing five deaths and millions of dollars in damage (Sheldon Lake Drainage Improvement Project 2002, 2003a, b; Endres 2004).

The effects of urban runoff after major storms on receiving-aquatic organisms or other beneficial uses are very site-specific. Different land-development practices create substantially different runoff-flow characteristics. Different rain patterns cause different particulate washoff, transport, and dilution conditions (Laws 1993). Land use in the drainage catchment area of Sheldon Lake is medium to high density residential with arterial streets, schools, businesses, houses and apartments, serviced to about 70% by curb, gutter and storm sewers, and 30% by roadside ditches and culverts. Soils in the study area were considered to have low infiltration and high runoff potential. During November 2002 Sheldon Lake was drained and allowed to dry out for the month of December while waterfowl and approximately 21 000 fish were relocated. This period was chosen because fish then tended to aggregate making capturing easier, oxygen stress on the fish are also minimized in winter, and furthermore are freezing temperatures ideal for removing bottom of sediment. The project began December 16, 2002 and was completed by June 23, 2003. During this time 41 285 m^3 of lake bottom sediment was removed and 746 m of storm sewer pipe, 457 m of box culvert, 274.32 m of water line and nine stormwater inlets installed (Sheldon Lake Drainage Improvement Project 2002, 2003a, b).

After completion of the project Sheldon Lake was refilled and stocked during the summer of 2003 with largemouth bass *(Micropterus salmoides*), bluegill sunfish (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*), crappie (*Pomoxis annularus*) and channel catfish (*Ictalurus punctatus*). Due to the eutrophic history of

Lake Sheldon, artificial mixing was introduced. The mixing is provided by two air compressors connected with a network of tubes and air bubble aerators in order to prevent growth of bloom-forming cyanobacteria. To regulate Sheldon Lake water depth variation in the summer and fall, adding water is supply by Pleasant Valley, Cache la Poudre River and the Colorado-Big Thompson watershed (Sheldon Lake Drainage Improvement Project 2002, 2003a, b).

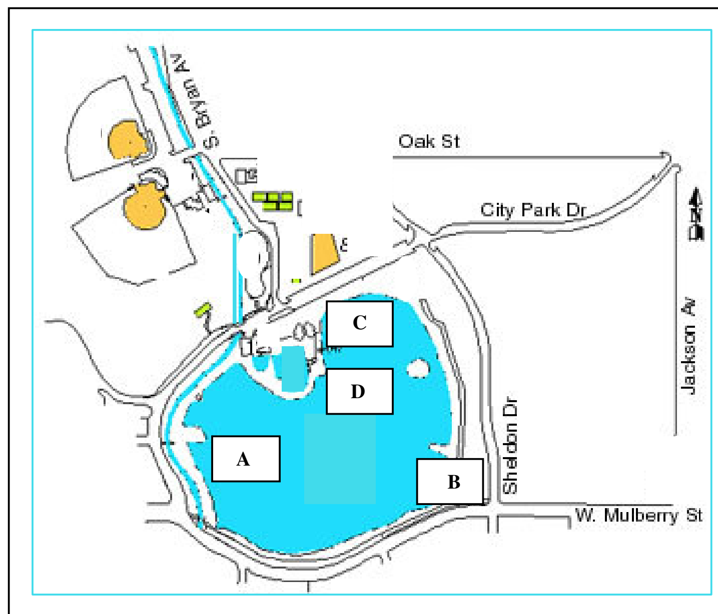

Figure 4.1 Map of Sheldon Lake and City Park recreational area, Colorado (Sheldon Lake Drainage Improvement Project 2002) (Scale: 10 m = 5 mm).

4.2.2 Sampling Protocol

Sampling was performed at four sampling stations at different depths and was carried out in the morning (8.00-9.00) and afternoon (16.00-17.00)(Fig. 4.1). According to Bottrell *et al.* (1976) and Cobelas and Arauzo (1994), the most ideal sampling intervals for plankton communities should be less than their generations time, which

can be very short, and call for intensive sampling protocols. The use of daily, or at least weekly, samples appears to be a reasonable compromise, but this was not possible during this study and samples were taken every three to four weeks. Most field observations show more than 1-3 dominant species at any phase of seasonal development as predicted by the competitive exclusion theory (Hardin 1960). The reasons are found in the different responses of phytoplankton on the frequency of disturbances or changes in abiotic resource conditions at different scales (Reynolds 1984). These different scales are (1) shorter than one generation time induce physiological responses, (2) frequencies between 200 and 20 h interact with the phytoplankton growth rate, and (3) disturbances at up to 10 days intervals can initiate a successional sequence in phytoplankton development. Due to our three to four weekly sampling frequencies, we confined our analyses to successional sequence in phytoplankton development and influencing abiotic factors. Most samples were analysed within one week after collection. Species composition and community structure were assessed from 1 mL aliquots sampled from 200 mL vertical water samples, then fixed with buffered 5% (v/v) formaldehyde. Phytoplankton cells were identified and counted from transects in a Sedgewick-Rafter sedimentation chamber using an inverted microscope. Collection, handeling, identification and counting of phytoplankton were done following the procedures of Lund *et al.* (1958) and Padisak (1993). Zooplankton was sampled by means of two net hauls (mesh size, 80 µm) from bottom to surface. The two samples were pooled and preserved in formaldehyde (2% (v/v) final concentration). We measured the length of as many animals as required to ensure that the standard error was < 10% of the mean length of each species. Abundance was determined from length-weight regressions (Downing & Rigler 1984).

An Ekman grab sampler (Brower & Zar 1977) was used to collect samples of benthic macroinvertebrates on a monthly base at all four sampling sites. Organisms were futher sampled on the submerged stems of macrophytes at sampling sites A and B by scraping. These benthic samples were then preserved with 70% (v/v) ethanol, and stored at 4 °C before they were sorted. Organisms were pooled together from all four sample sites to form an aggregate sample unit. Samples were washed through a 0.25 mm mesh sieve and retained animals were carefully separated from detritus. The macroinvertebrates were identified according to Pennak (1978) and Merritt and Cummins (1996). Specimens were identified to the lowest possible taxonomic category and counted using a dissecting microscope. The classification method of Cummins (1974) was used to classify the different macroinvertebrates into functional feeding groups, since it has been suggested that the bioassessment of water quality based on functional feeding groups of macroinvertebrates may be superior to that based on community structure, because it reflects more ecologically significant attributes of freshwater systems (Rabeni *et al*. 1985).

Birds were sampled monthly from shore with a x 45 spotting scope. A running record of time, location, and behaviour was kept for all individual birds encountered to decrease the likelihood that individuals were counted more than once. For community analysis, we used only non-passerine birds that feed at or beneath the surface of the water. We chose these species because they are most strongly and unambiguously linked to lake characteristics, and were most reliably visible and identifiable by sight alone. Classification of the different bird species was done using the Colorado breeding birds' atlas (Kingery 1998).

Chl*a* was extracted from lyophilized GF filters using N, N-dimethylformamide for 2 hours at room temperature. Chl*a* was measured photospectrometrically at 647 and 664 nm, respectively and calculated according to Porra *et al.* (1989). Standard analytical techniques were used for all the chemical and physical variables. Nutrients, dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) were analyzed using classical spectrophotometric methods (American Public Health Association, American Water Works Association, and Water Pollution Control Federation 1980). Temperature profiles and pH of the water column were measured with an YSI model 2100 thermometer and a 211 microprocessor pH meter at all four sampling stations. Data of wind speed, direction and rainfall was measured at the meteorological station of the Colorado State University, 2 km away from the lake. Fish species were collected from hook and line anglers, as well as with capturing gear that consisted of monofilament nylon nets with a 6.25 mm mesh size. Nets were set in late afternoon close to shore and macrophyte patches of sampling site A and was retrieved early the following morning. Classification of the different fish species was done using the field guide to freshwater fish (Schultz 2004).

The trophic state of Lake Sheldon was based on a combination of the classification of Carlson (1977) and Willen (2000). Freshwater lakes all over the world exists from eutrophic (rich in nutrients), to oligotrophic (low nutrient levels), while in between states also exist e.g., mesotrophic. In general, the amount of Chl*a*, cell density, and /or type of algal species present can be used as indicators of the trophic status of the water, so long as no toxic compounds that inhibit algal growth are present. Other factors have also to be taken into account, such as temperature and pH (Premazzi &

Chiaudani 1992). In our study, we used algal composition, secchi depth, total phosphorus and Chl*a* as indicators of the trophic state of Sheldon Lake a year after restoration. The dominant species shown in Table 4.1 are associated with corresponding water types (Willen 2000).

Table 4.1 Dominant species associated with water of different trophic levels (Modified from Willen 2000).

OLIGOTROPHIC	
Chlorophyceae	[*Staurastrum* sp., *Gleoocystis* sp., *Spaerocystis schroeteri*]
Diatoms	[In Europe, *Cyclotella* associated with other species such as *Fragilaria* sp., *Synedra* sp., *Dynobrion* sp. and *Melosira* sp.while in North America lakes, *Asterionella* sp., *Tabellaria* sp.and *Melosira* sp.associated with *Dynobrion* sp.]
Crysophytes	Associated with very low nutrient levels [*Dynobrion* sp., *Mallomonas* sp., *Synura* sp.]
Chlorococcales	Some lakes may be dominate by *Oocystis* sp.
Dinoflagellates	[*Ceratium hirundinella, Peridinium incospicuum* and *Peridinium willei*]
MESOTROPHIC	
Dinoflagellates	[In Finnish lakes, *Ceratium* sp., *Gymnodinium* sp., *Peridinium bipes* and *Peridinium cinctum*]
Chlorococcales	[*Pediastrum* sp., *Scenedesmus* sp.]
Chlorophyceae	[*Staurastrum gracile*, *Straurastrum pingue,* and *Straurastrum planctonicum*]
EU/HYPERTROPHIC	
Cyanophyceae	[*Microcystis* sp., *Anabaena* sp., *Aphanizomeno*n sp. and *Lyngbya* sp. and *Oscillatoria rubescens*]
Euglenophyceae	[*Euglena* sp.]
Chlorococcales	[*Cosmarium* sp.]
Diatoms	[*Asterionella* sp., *Fragilaria* sp., *Synedra* sp., *Melosira* sp.]

We also applied Carlson's trophic state index approach to classify the trophic state of the lake that is based upon the calculation of an index with a range between 0-100 (Table 4.2). The theory of the index is based upon the statistical relationships between phosphorus loading, phosphorus concentration, chlorophyll and transparency, by using $\log_2$ of the secchi disc transparency as the starting point. Zero is set at 64 m, the integer greater than the maximum transparency ever recorded [42 m for Lake Masyuko in Japan (Hutchinson 1957)], and each halving of transparency increases the index by 10. Chlorophyll and total phosphorus concentration were related to transparency by regression equations and then added to the scale in Table 4.2. (Carlson 1977). An index category of less than 20 represents ultra-oligotrophic, 30-40 oligotrophic, 45-50 mesotrophic, 53-60 eutrophic and above 70 hypertrophic (Kratzer & Brezonik 1982).

Table 4.2 Carlson's Trophic State Index and its associated parameters (Carlson 1977).

TSI	Secchi disc depth(m)	Total phosphorus(µg/L)	Chlorophyll(µg/L)
0	64	0.75	0.04
10	32	1.5	0.12
20	16	3	0.34
30	8	6	0.94
40	4	12	2.6
50	2	24	6.4
60	1	48	20
70	0.5	96	56
80	0.25	192	154
90	0.12	384	427
100	0.06	768	1183

4.2.3 Data analyses

The results were recorded on standard Excel spreadsheats for data processing, and statistical analysis was performed using SYSTAT® 7.0.1 (1997).

4.3 Results

4.3.1 Phytopankton

The phytoplankton succession in Sheldon Lake *per se* provides a valuable model for the changing environment in a restorated, artificial mixing shallow urban lake. The mid and late summer phytoplankton community composition and structure of 2004 did not vary much. Cyanobacteria were the most abundant group of phytoplankton across all sampling sites, followed by Chlorophyceae, Bacillariophyceae and Euglenophyceae (Fig. 4.2).

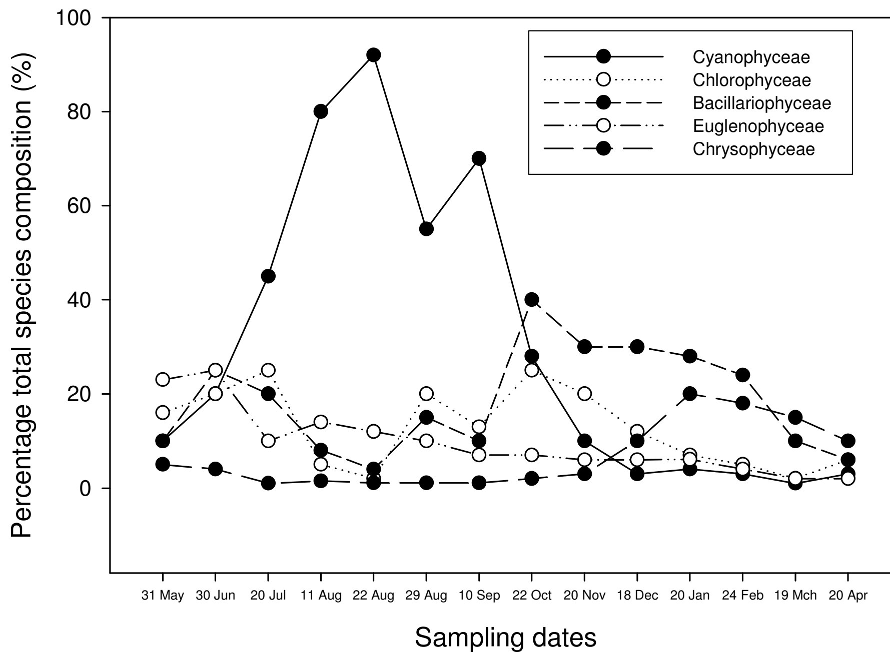

Figure 4.2 Seasonal changes and contribution to phytoplankton species composition as percentage for the five major algal classes 2004-2005 from the four sampling sites.

The dominant cyanobacteria species were *Microcystis aeruginosa* and relatively less abundant was *Woronichinia naegeliana* while *Microcystis viridus* were less abundant to *Microcystis aeruginosa* in 2001 without mixing (Table 4.3, Fig. 4.3). These species disappear from the scene in early October after a period of high winds and heavy rainfall. A shift to a mostly Bacillariophyceae dominated community was observed due to high silica concentrations (silica 6.3 mg/L) and resuspension events in autumn 2004. Diatoms appeared to be more tolerant than summer species like cyanobacteria to reduced light doses and rapid fluctuations in irradiance (Reynolds 1984).

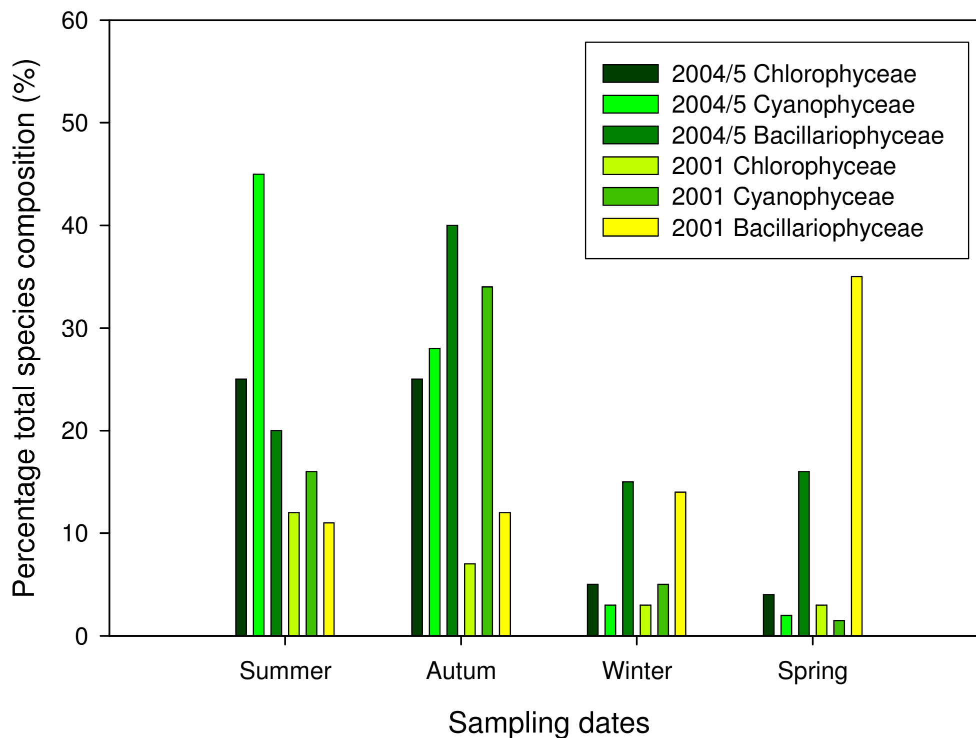

Figure 4.3 Seasonal changes and contribution to phytoplankton species composition as percentage for the Chlorophyceae, Cyanophyceae and Bacillariophyceae in 2001 and then 2004-2005 from the four fixed sampling sites.

During the winter of 2004-2005, reduce temperatures, light energy input as well as silica limitation led to lower primary production and a decline in diatom biomass which were replaced by Chrysophyceae during the clear water phase. Towards late spring Chlorophyceae became dominant after the clear water phase, when the phytoplankton biomass declines rapidly to very low amounts (Fig. 4.2). However this was not the case during the spring of 2001 without mixing when *Melosira varians* were abundant. The concentration of total phosphorous (TP) and Silica increased significantly from 2001 without mixing to 2004-2005 with mixing, while Secchi-depth decreased correspondingly (Table 4.3).

Table 4.3 Annual mean values and ranges for selected variables for the years 2001 and May 2004 to April 2005 (Data for 2002-2003 is not available due to restoration; ± indicate standard error).

Varaible (unit)		**Year**	
	2001* Sheldon Lake before restoration (n=4)	**2004-2005 Sheldon Lake one year after restoration (n=24)**	**2004-2005 Inflow from Urban run-off inlets and Cache la Poudre River (n=20)**
Secchi depth (m)	0.51 (0.31-0.70)	0.61 (0.25-0.80)	Nd.
pH	8.5	8.4	8.1
SRP (μg/L)	Nd	4 (0-16)	3 (0-22)
Ammonia (mg/L)	Nd	0.467 (± 0.139)	0.302 (± 0.100)
Total Nitrate (mg/L)	2.280 (± 0.542)	2.87 (± 0.046)	2.47 (± 0.046)
Total Phoshorous (mg/L)	1.81 (± 0.066)	2.21 (± 0.061)	1.40 (± 0.161)
Silica (mg/L)	4.9 (± 0.052)	6.3 (± 0.031)	Nd
Conductivity (ms/cm)	Nd	1.34 (± 0.12)	1.73 (± 0.21)
DO (mg/L)	Nd	8.3 (± 0.4)	10.7 (± 0.7)
BOD (mg/L)	Nd	6.8	3.3
Chlorophyl*a* (μg/L)	72 (6-420)	58 (10-693)	Nd
Dominant phytoplankton spp.	*Microcystis aeruginosa; M. viridus* (summer) *Asterionella formosa* (winter)	*Microcystis aeruginosa; Woronichinia naegeliana* (summer) *Melosira varians*, *Asterionella formosa* (winter)	Bacillariophyceae (summer and winter; only for Cache la Poudre) Urban runoff (Nd)

Nd = not determined
n = sample times per year
* - Unpublished data and personal communications by B. Whirty, Park Operations Supervisor, City of Ft. Collins, and S. Seal, Fish health, Environmental Sciences, CSU

The secchi depth was quite shallow during summer 2004-2005 with an average of 0.25 m during a peak of cyanobacteria (i.e. *Woronichinia naegeliana* and *Microcystis aeruginosa*) blooms from August to September (Table 4.3). There also was a strong negative correlation between Chl*a* and the secchi depth during this time indicating that the dense blooms of cyanobacteria significantly reduced light penetration through the water column at the sampling sites (Fig. 4.4).

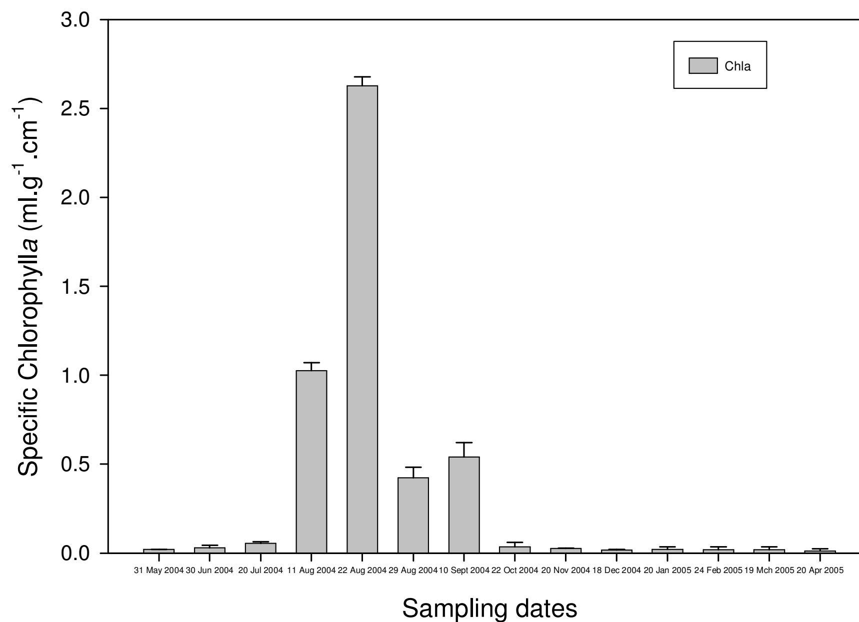

Figure 4.4 Specific Chl*a* measured from May 2004 to April 2005, Sheldon Lake, Colorado. Error bars represent standard deviation from the mean value.

The classification of the lake's trophic state one year after restoration, using the Carlson (1977) trophic state index and its associated parameters as well as the phytoplankton indicator concept of Willen (2000), indicated that Sheldon Lake is eutrophic to hypertrophic according to the following parameters taken during summer: Secchi disc depth (0.25m); Total Phosphates between 0.2 mg/L to 0.3 mg/L; Total Nitrogen between 0.2 mg/L to 0.4 mg/L; Chlorophyll 0.693 mg/L; Silica 6.3

mg/L. Dominanted phytoplankton indicators: *Asterionella* sp., *Synedra* sp., *Melosira* sp., *Cosmarium* sp., *Euglena* sp. and *Microcystis* spp.

4.3.2 Zooplankton

The abundances of herbivorous zooplanton (cladocerans; omnivorous copepods and rotifers) were close to zero in Lake Sheldon from June to the middle of July, when the abundances increased somewhat, although they never exceeded 10 ind/L. During August to the end of September numbers increased above 20 ind/L (results not shown). An interesting feature of the zooplankton was the relationship between cladocerans (*Bosmina* sp.) and a cyanobacterial bloom of *Microcystis aeruginosa*. During dense surface blooms of cyanobacteria in August to the end of September, there was a 5-fold higher density of *Bosmina* sp. at sampling site C which contains high numbers of cyanobacterial colonies than at site D were cyanobacterial colonies were absent (Fig. 4.5).

At the end of September there were a decline in abundance of cyanobacteria and *Bosmina* sp. due to a temperature drop and storm weather activities. However, zooplankton community was dominated for most of the summer by the small-bodied *Bosmina* sp., which provides little potential for the removal of significant amounts of phytoplankton. This contrasts with the situation in spring during the clear water phase, when a relatively high population density of larger cladocerans (*Daphnia magna*) occurs.

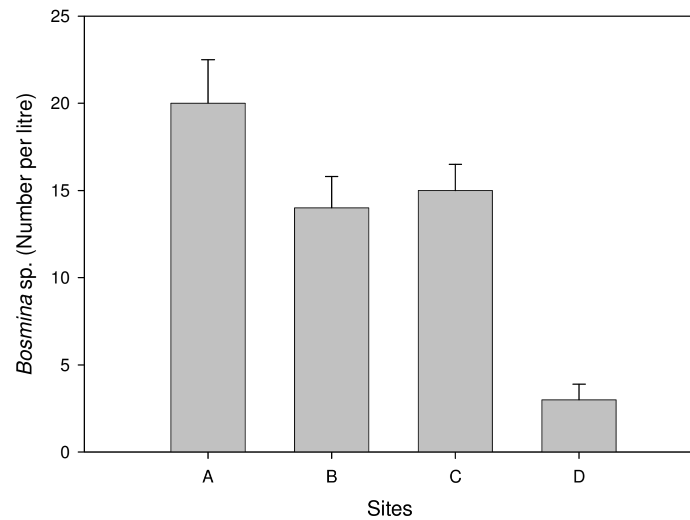

Figure 4.5 Abundance in *Bosmina* sp. during summer 2004 (A) Site with cyanobacterial surface bloom and sparse macrophyte growth; (B) Site with cyanobacterial surface bloom and sparse macrophyte growth; (C) Site with cyanobacterial surface bloom but without a macrophyte growth; and (D) Site without cyanobacterial surface bloom and macrophyte growth. Error bars represent standard deviation from the mean value.

4.3.3 Benthic macroinvertebrates

Fluctuations in lake levels due to variable seasonal inflow of stormwater runoff, as well as the short time after removing of bottom sediment for restoration purposes, give rise to a very unstable and ill-defined littoral zone, with negligible development of a littoral flora.

The most abundant macrophyte recorded were *Typha angustifolia* at sampling sites A and B. Macroinvertebrate diversity in a sampling programme carried out monthly were very low and most of the lake were dominated by Nematoda, Oligochaete worms together with chironomid larvae of the genus *Chironomus* (Table 4.4).

Table 4.4 Dominant taxa in each functional feeding group.

Functional feeding group	Taxa
Collector-gatherers	Chronomidae, Oligocheata, *Tricorythodes minutus*, *Acentrella insignificans*, *Baetis* spp.
Collector-filterers	*Cheumatopsyche* spp., *Simulium* spp., *Hydropsyche occidentalis*, *H. cockerelli*, *H. morose*
Scrapers	*Hydroptila pecos*, *Petrophila avernalis*
Shredders	*Caecidotea cummunis*, *Oecetis inconspicua*
Microphyte-piercers	*Hydroptilia pecos*
Predators	Nematoda, *Acari, Dugesia dorotocephala, Oecetis inconspicua*

Oligochaetes and chironomids were the most abundant at sampling site A followed by collector-filterers, scrapers and shredders which were particularly poorly represented at sampling sites C and D (Fig. 4.6).

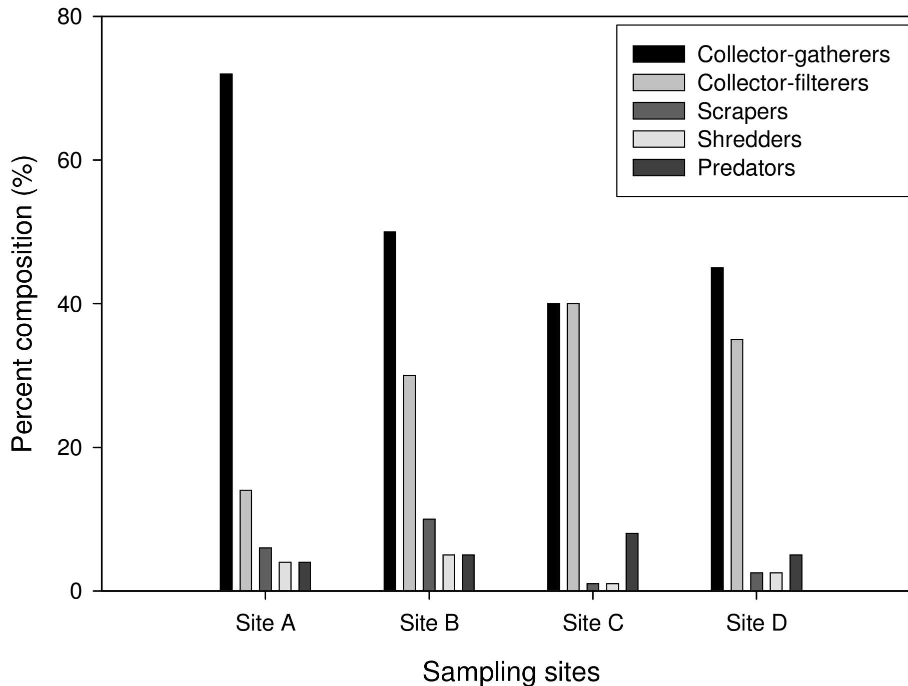

Figure 4.6 Macroinvertebrate abundance (%) at the four sampling sites, Sheldon Lake, Colorado during summer 2004.

4.3.4 Vertebrates

With the removal of the bottom sediment and restoration of the lake, the fish fauna were restock in 2003 by the Colorado Division of Wildlife and included the following species: 3 000 largemouth bass (*Micropterus salmoides*); 3 000 bluegill sunfish (*Lepomis macrochirus*); 12 000 crappie (*Pomoxis annularus*); 3 000 channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*). Sufficient specimens of two of these species (largemouth bass and bluegill sunfish) were collected during the investigation to allow for a meaningful analysis of the stomach contents.

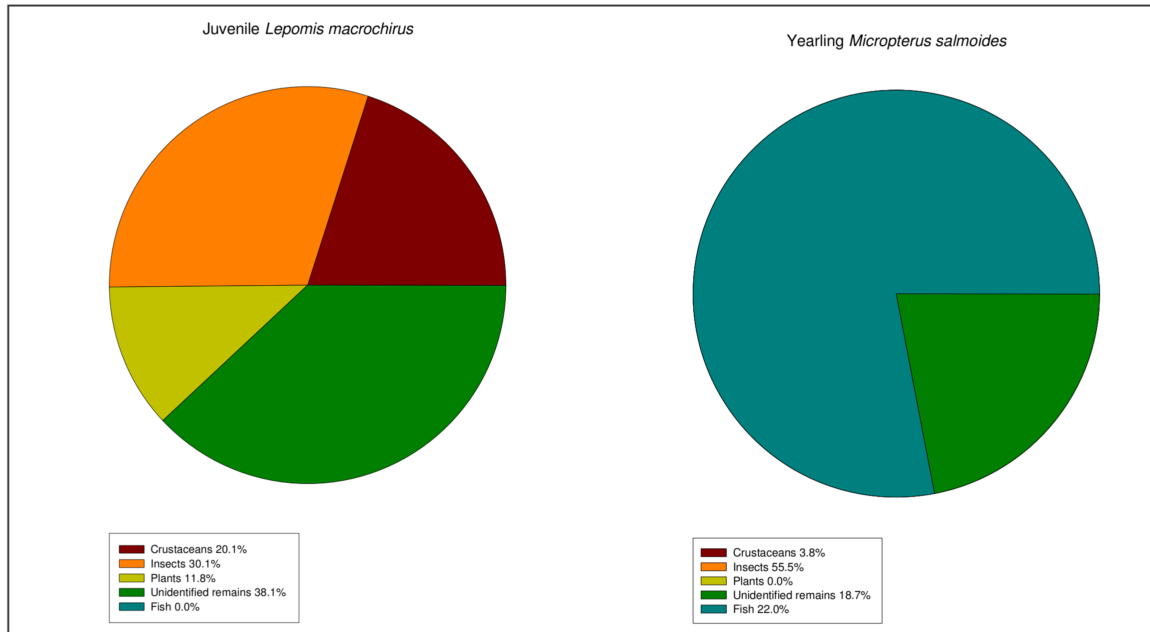

Figure 4.7 Composition by volume of the gut contents of juvenile *Lepomis macrochirus* and yearling *Micropterus salmoides* of Sheldon Lake, Colorado during summer 2004 (n = 50).

Figure 4.7 shows the percentage of the volume of the stomach contents contributed by different food items. The fish were mainly juveniles' bluegill sunfish and yearling largemouth bass, with modal lengths varying between 20-200 mm. While zooplankton

was found in the stomachs of all the species that were dissected, macroinvertebrates were the most important food type to the juvenile bluegill sunfish that was caught in the littoral zone of sampling sites A and B where macrophytes were sparce.

4.3.5 Waterbirds and nearshore birds

The monthly count and species number of waterbirds from May 2004 to April 2005 is reported in Figures 4.9a and b. A year's total count was 13 species and an average of 65 individuals per month. During this time the following bird species were observe: double-crested cormorant (*Phalacrocorax auritus*); back-crowned night-heron (*Nycticorax nycticorax*); great blue heron (Ardea *herodias*); spotted sandpiper (*Actitis macularia,*); black tern (*Chlidonias niger*); ring-billed gull (*Larus delawarensis*); canada goose (*Branta canadensis*); mallard (*Anas platyrhynchos*); northern shoveler (*Anas clypeata*) (Fig. 4.8); bufflehead (*Bucephala albeola*); common goldeneye (*Bucephala clangula*); common merganser (*Mergus merganser*) and the hooded merganser (*Lophodytes cucullatus*).

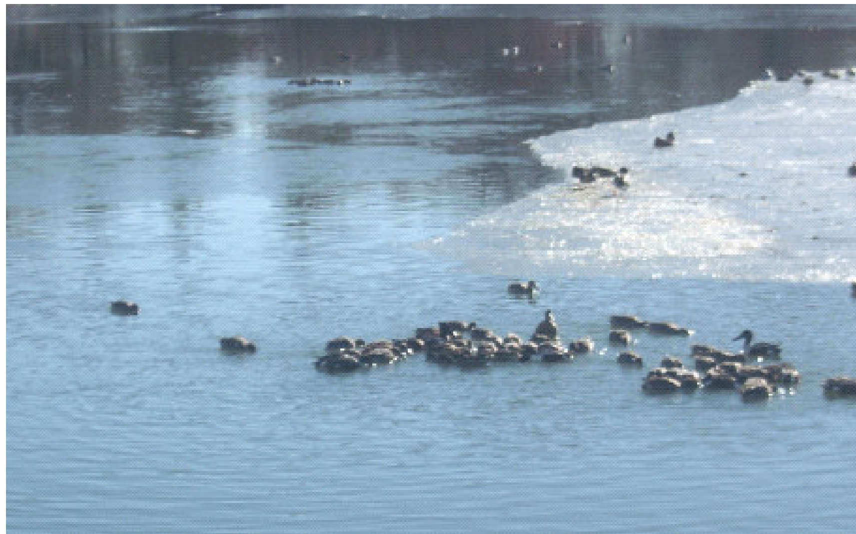

Figure 4.8 Northern shoveler's clustered around air bubbles that form surface 'boils' in the ice cover during artificial mixing.

The number of birds was high during the winter, but the diversity was higher during the summer months with the exception of the duck species with a higher diversity during the winter months. The most dominant birds during our survey included mallards (*Anas platyrhynchos*), Canada goose (*Branta canadensis*) and the ring-billed gull (*Larus delawarensis*). The number of sandpipers remained quite small throughout the year while the number of predator birds increased during the summer, which was probably a result of the increase in benthic biomass.

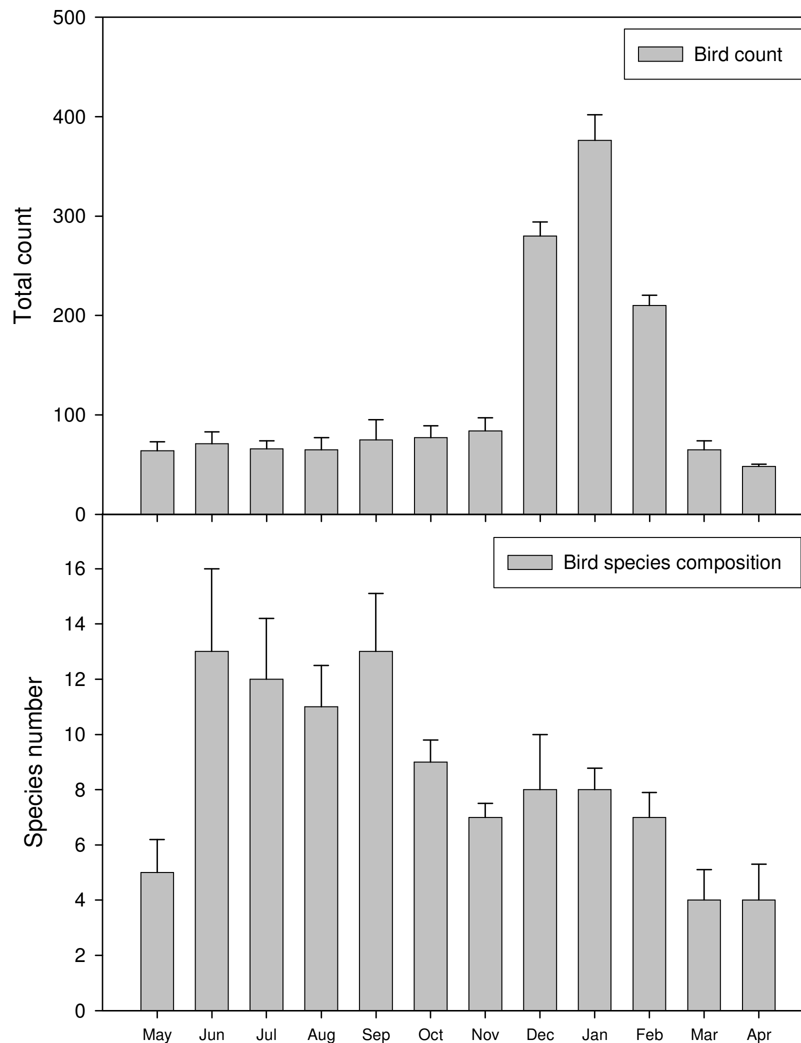

Figure 4.9 Monthly distribution of waterbirds and nearshore birds in Sheldon Lake, Colorado (a) count and (b) species. Error bars represent standard deviation from the mean value.

4.4 Discussion

4.4.1 Phytoplankton

Phytoplankton communities in the water column of temperate lakes undergo significant changes within individual years. These changes have been termed as 'seasonal succession' by plankton ecologists. Seasonal succession of phytoplankton has more similarities with the succession of terrestrial vegetation than with its seasonal aspect. Many generations are involved with several quite predictable and distinct phases. On the scale of generation times, the several months in plankton succession corresponds to tens of years in terrestrial grassland, and to centuries in forest succession. Under favourable physical conditions the intrinsically transient nature of early and mid-successional phases, and the selfsustainability of final stages can be shown. It is only the external cycle in climatic and hydrological conditions that forces phytoplankton succession to restart each year (Sommer 1991). The phytoplankton composition in 2004-2005, especially the bloom of cyanobacteria in the second half of the year is an indication of the eutrophic to hypertrophic state of Sheldon Lake.

A shift from cyanobacterial dominance to a phytoplankton community dominated by green algae and diatoms has been observed in artificially mixed lakes or reservoirs (Symons *et al*. 1970; Haynes 1973; Hawkins & Griffith 1993; Cowell *et al*. 1987). However, this was not the case in our study since cyanobacteria dominated the phytoplankton community the whole summer of 2004. The predominance of cyanobacteria can be due to the fact that the artificial mixing velocity was not

sufficient on sunny, calm days to keep *Microcystis* entrained in the turbulent flow in the entire lake. Other factors that could have determined the insufficiency of the artificial mixing were the depth of the lake where the aeration tubes were installed, and the distribution of the tubes over the lake area (Cooke *et al*. 1993). The failure to reduce dominance by colony-forming cyanobacteria can often be ascribed to insufficient mixing velocities and/or a low mean depth of the lake or reservoir (Knoppert *et al*. 1970; Lackey 1973; Osgood & Stiegler 1990). Other cyanobacteria in Sheldon lake with mixing, during the summer of 2004-2005 were *Woronichinia naegeliana* while *M. viridus* where abundant in 2001 without mixing.

Detailed analyses on the summer succession of Lake Sheldon in 2004 have shown that the meteorological (rain and wind) and chemical conditions (Silica and total Phoshorous), especially the frequency of storms and a drop in temperature, have a fundamental effect on phytoplankton succession. These periodic events together with the artificial mixing did enable cyanobacteria to maintain high population density during the late summer. This observation is supported by the variable physical and morphometric characteristics, such as those typical of many urban water bodies. These characteristics can play a role in influencing phytoplankton community composition, which include the following: mixing patterns (Harris & Trimbee 1986), high wind events (Carrick *et al*. 1993), flooding (Van den Brink *et al*. 1994), water level fluctuations (Garcia de Emiliani 1997) and changes in flushing rate (Bailey-Watts *et al*. 1990). The diatom peak (*Synedra rumpens var fusa* and *Melosira varians*) in October of 2004 that succeeded the cyanobacteria peak (*Microcystis aeruginosa*) in summer was correlated strongly with resuspension events caused by meteorological

activities (rain and wind) on the 4, 21, 25 and 27th of September 2004 when wind velocity reach a maximum of 10.2 m/s.

The most dominant species found with mixing during the winter months of 2004-2005, were *Melosira varians* and *Asterionella formosa*, which occur, in significant abundance under the ice cover. These phenomena correlated with findings of Agbeti and Smoll (1995). *Melosira varians* were abundant only in spring of 2001 without mixing, while they where abundant in the winter of 2004-2005 with mixing (Fig. 4.3). The fact that the winter season showed a consistent positive relationship between water temperature and algal biomass is expected, because most phytoplankton species for e.g., *Microcystis aeruginosa* reached their optimal growth rate in the range of 20-25 °C (Reynolds 1984). However during spring, summer and autumn periods other factors, such as grazer-, nutrient- or light-limitation tend to play an important role, while these factors seems less important during winter periods in Sheldon Lake. Therefore, the direct effects of temperature ranging between 1-4 °C on phytoplankton growth and reproduction become relatively more significant. Although there is little experimental evidence that shows diatom species dominate in winter and spring at temperatures below 5 °C, because of their higher growth rate in colder temperatures compared to cyanobacteria or Chlorophyta (Lund 1955; Foy & Gibson 1993). The Chlorophyceae were much higher in 2004-2005 with mixing compared to 2001 without mixing (Fig. 4.3).

Foy and Gibson (1993) have demonstrated in culture experiments on three planktonic diatom species that growth rate show a progressively decreasing response to increasing temperature above 10 °C. During the clear water phase of 2005 the most

common species were *Dinobryon suecicum* and *Chrysococcus minutes*. These Chrysophytes species tended to dominate only in conditions of low productivity and low to moderate pH. They were replaced by the end of spring with chlorophytes species with a generally higher nitrogen optima and the ability to utilize bicarbonate in photosynthesis, as well as to thrive in a higher pH environment (Sandgren 1988; Reynolds 1998; Levine & Schindler 1999). The reduction of *Daphnia magna* at the end of the clear-water phase may be the result of higher transparency when the phytoplankton biomass was low, and this may have increased the predation risk from visual hunting fish. Furthermore an increase in the N:P ratio during this time is associated with waste material of free-living waterfowl, which has assembled, in great numbers at surface 'boils' produced by artificial mixing. The artificial mixing created open areas in the ice-covered lake during the winter months of December and January. Most of these waterfowl has migrated from other neigbouring ice covered lakes where food was limited.

4.4.2 Zooplankton

Results of laboratory studies (Lampert 1987) suggest that many colonial cyanobacteria are either not eaten or are a poor food source for large zooplankton, particularly *Daphnia*. Therefore, at times when these colonial forms dominate the phytoplankton, *Daphnia* populations might be expected to show decreased growth and fecundity in response to food limitation or toxicity. However, the theory was supported by our field studies in 2004-2005 when we observed that the *Daphnia* population decreased during cyanobacteria bloom periods probably due to the combined impact of fish predation and food limitation and was replaced by *Bosmina*

sp. This seasonal pattern is in agreement with other the observations (McNaught 1975; Gliwicz 1977; Beaver & Crisman 1982). The observed increase in *Bosmina* sp. in Sheldon Lake during this time may be related to its ability to avoid predators by positioning in the cyanobacterial surfaces blooms that provide temporary refuge in the absence of macrophytes.

A year after the removal of the bottom sediment of the lake for restoration purposes, macrophytes and submerged vegetation in Lake Sheldon were sparse and only a few patches of the macrophyte, *Typha anustifolia* was prominent at the littoral zone of sampling sites A and B. This correlate strongly with the observation of Moss *et al*. (1986), who found failure of submerged plant colonization of the majority of the open water in the Coockshoot Broad after bottom sediment removal in 1982. The failure of plant colonization is not yet understood but may be due to the lack of sufficient inoculums of plant material or simply to the fact that phytoplankton populations are still preventing adequate light transmission. De Bernardi and Giussano (1990) also observed that in shallow lakes where plants have been lost, circumstances leading to cyanophyte dominance also make restoration of the plant communities difficult because cyanophytes are often indigestible or nutritionally poor for invertebrate grazers. Macrophytes fill multiple roles in the ecosystem function (Carpenter & Lodge 1986) and in the mediation of predator-prey interactions involving fish and macroinvertebrates (Crowder & Cooper 1982; Savino & Stein 1982). Investigators have suggested that macrophytes provide a refuge to cladocerans from fish predation and thus contribute to biomanipulation efforts to reduce phytoplankton-standing stock (Timms & Moss 1984). Scheffer *et al*. (1993) suggested that macrophyte refuges for

Daphnia contribute significantly to the stability of the high *Daphnia*-low phytoplankton-high macrophyte state in shallow lakes.

Zooplankton in shallow, non-stratified lakes does not have the option of moving down to the metalimnion and hypolimnion to avoid predation. However, studies in shallow lakes have shown that zooplankton aggregate in nearshore areas among the structurally complex macrophyte beds during the day. For example, the density of *Daphnia magna* was 20-fold higher within the macrophyte beds during daytime than during night-time (Lauridsen & Buenk 1996), indicating that horizontal migration between the structurally complex macrophyte beds and the open water may be a way to reduce the risk of predation by planktivores in lakes where vertical migration is restricted. In parallel to vertical migration, zooplankton exposed to chemical cues 'i.e. kairomones'from fish also increases their use of the macrophyte habitat (Lauridsen & Lodge 1996). The data correlates with Schriver *et al*. (1995) that *Daphnia* tolerate a higher fish density in macrophyte-rich lakes and that the presences of submerged vegetation may further enhance grazer control. We proposed that there is a compensatory interaction between cyanobacteria and zooplankton in shallow lakes where floating leaved macrophytes and submerged vegetation is scarce. The zooplankton may prevent intense predation by zooplanktivorous fish by using cyanobacteria blooms as temporary refuge in the absence of macrophytes.

It was also found in previous studies that macrophyte densities could affect chemical fate processes by increasing the surface area available for sorption of hydrophobic compounds. Caquet *et al*. (2000) reported the presence residues of deltamethrin and lindane in the macrophyte samples 5 weeks after treatment but never in the sediment.

Macrophytes can also affect physicochemical composition in surrounding waters, influencing the distribution and community structures of many aquatic organisms (Barko *et al*. 1988). In addition, macrophytes provide three-dimensional structure within constructed ecosystems, which affect organism distribution and interactions. Others have shown that macroinvertebrate community diversity is influenced by patchy macrophyte abundance and specific macrophyte types (Schramm *et al*. 1987; Learner *et al*. 1989). Cladoceran communities are not only associated with macrophytes for refuges but also graze on the periphytic algae on aquatic macrophytes (Campbell & Clark 1987). The impact of the low to zero densities of macrophytes in Sheldon Lake may cause indirect effects on organisms by influencing trophic linkages such as predator-prey interaction between invertebrates and vertebrates.

4.4.3 Macroinvertebrates

The differences in dominant taxa of macroinvertebrates in terms of production among sampling sites in 2004-2005, suggest that these differences might be related to the variations in physicochemical and food-related variables. The numbers of Oligochaetes and chironomids, that significantly increase in abundance in the soft bottom sediment at sampling site A, may probably be due to the exposure to organic pollution at the inlet of a urban runoff pipe situated in the sampling area (Fig. 4.6). These taxa contain many generalist feeders that benefit from increased organic loading. Other studies have also shown that the increased Oligochaetes and chironomid population densities were in association with organically polluted lotic systems (Prat & Ward 1994; Zamora-Munoz & Alba-Tercedor 1996). Wiederholm (1984) also reported that the presence of Oligochaeta, *Physella* sp., *Simulium* sp., and

a dominance of Chironomidae genera, was characteristic of aquatic systems affected by organic pollution.

The distributional patterns of the dominant taxa in each of the functional feeding groups which reflect resource distribution and use, and facilitate the understanding of organic matter processing in a freshwater ecosystem like Sheldon Lake were as followes: Collector-gatherers (Chironomidae, Nematoda, Oligochaeta); Collector-filterers (*Simulium* spp.); Scrapers (*Physella* sp.); Shredders (*Caecidotea cummunis*), Predators (Acari) (Fig 4.4). The density of the collector-filterers were the highest in November and May at the standing stock of macrophytes (*Typha angustifolia*) of sampling sites A and B, which created more surface attachment sites for collector-filterers, such as simuliids. The macrophytes at these sampling sites overwinter as rhizomes. Although the ability to absorb nutrients from the sediment and light from above the water surface make emergent macrophytes (*Typha angustifolia*) competitively superior, it also restricts them to shallow water, making emergent macrophytes a less dominant primary producer. A higher abundance of scrapers were observed in the fall and summer 2004 when higher water temperature and light intensity stimulate primary production and resulted in an increase in the food resources, especially during the increase of the diatom population in late summer. Higher densities of shredders occur in fall 2004 during a period of maximum availability of deciduous leaves. Predators showed a variation in density, with the highest densities during summer and fall. Due to the fact that sampling sites C and D did not support any macrophytes that create more diverse habitats for the attachment of benthic macroinvertebrates, the abundance and species richness were very low at these sites. On the contrary, the filamentous green alga, *Cladophora* sp. was found in

low numbers at all of the four sampling sites. Moreover, Steinman (1996) indicated that *Cladophora* sp. is not utilized as a food resource by macroinvertebrate scrapers because they are too large to graze. In addition, numerous *Caecidotea cummunis* were collected in the filamentous alga *Cladophora* during our survey. Macroinvertebrate ecology in Sheldon Lake supported the concept of Kerans and Karr (1994) which stated that the direction of change with increasing human impacts would increase proportions of individuals comprising of collector-gatherers and collector-filterers, and decrease proportions of scrapers, shredders and predators.

In contrast with Sheldon Lake, lakes that retain submerged plant populations are more diverse and contain several mollusks species (Mason & Bryant 1975). Wortley (1974) demonstrated that this was a direct result of lack of habitat diversity and showed that when artificial plants constructed from polypropylene were introduced into the lakes without submerged macrophytes, large number of invertebrates established populations. Crayfish is one of the predators feeding on mollusks species in Sheldon Lake and account for a large proportion of the biomass (data not shown). These are not important only in terms of numbers and biomass but also because of their functional role in the ecosystem. On the other hand, crayfish may have negative effects on macrophytes, periphyton and mollusks especially if macrophytes are spares like in the case of Sheldon Lake (Lodge *et al*. 1994).

4.4.4 Vertebrates

On the basis of identifiable remains, macroinvertebrates and not zooplankton is the most important component of the diet of young non-piscivorous bluegill sunfish (*Lepomis macrochirus*) in Sheldon Lake. This phenomenon may be due to the

presence of piscivorous largemouth bass (*Micropterus salmoides*) resulting in change of habitat from the pelagic zone with no submerge macrophytes to the refuge of the littoral zone with sparse macrophytes. However, the juvenile bluegill sunfish had a strong negative effect on the density of macroinvertebrates, resulting in a smaller average size of macroinvertebrates. This observation agrees with previous reports that found that predators also affect the behaviour of their prey resulting in reduced activity or a change in habitat/ refuges (Werner *et al.* 1983; Werner & Hall 1988; Fuiman & Magurran 1994; Turner 1997). These predator-mediated changes in the habitat use of an intermediate consumer may affect its diet and thus change the direction of interactions in food chains.

4.4.5 Waterbirds and Nearshore birds

For aquatic birds, depth and size play an important role in shaping assemblages, but size appears to be the predominant factor. Differences in the composition of bird assemblages strongly paralleled differences in species richness and reflected species-area relationship reminiscent of those seen among aquatic bird communities in other geographical settings (Elmberg *et al.* 1993, 1994; Hoyer & Canfield 1994).

Small shallow lakes like Sheldon Lake (size: 6,07 hectares and max. depth: 1,4 meters) support only a core of widespread, generalist species simply because of their size, not because the water body is invariably shallow. A detectable but smaller effect of a lake's position in the landscape on its bird assemblage is also important, especially if it is situated in an urban environment like in the case of Sheldon Lake. Although birds are perhaps the best-studied taxon in human-dominated areas, our

understanding of the effects of human settlement on bird communities is in its infancy (Marzluff *et al*. 1998). Aerial piscivores which served as effective indicators of fish-assemblage type were relative scarce during our summer months survey of 2004, this may be due to the fact that the transparency of the water body was low because of cyanobacterial blooms (Secchi depth 0.25 m) and therefore not a profitable feeding site for aquatic birds that forage by surface plunging (Eriksson 1985).

During the winter months the birds may be a contributory factor to eutrophication when they assemble in great numbers at areas where artificial mixing provide openings in the ice-cover especially, the northern shoveler's which clustered around air bubbles that form surface 'boils' (Fig 4.8). This mixing method destroys or prevents thermal stratification and cause vertical circulation of phyto and zooplankton to the photic zone in the form of surface 'boils'. It is then an available food source for the northern shoveler's that filter the water through their 'Spoon-bill' ejecting the refuse through its 'sieve', and retaining whatever nutritious matter there may be. Their diet explains their preference for shallow, muddy areas that provide a bounty of free-swimming invertebrates (DuBowy 1996). A bald eagle (*Haliaeetus leucocephalus*) was frequently observed at sampling sites C and D during the winter months of December and January 2004-2005. The bald eagle is almost non-migratory, and only deserts its home during the coldest weather when the water is frozen. Artificial mixing ensure that a small percentage of Sheldon Lake at sampling sites C and D were not covered with ice during the winter months of December to January, this gave the bald eagle an opportunity for food. Occasionally the bald eagle was joined by crows and ravens when they were feeding upon carrion. On two occasions the bald eagle was spotted attacked waterfowl during January, and even killing a common goldeneye

duck at one of these occasions. Other birds frequently observed in trees near the lake during our survey included the belted kingfisher (*Ceryle alcyon*); northern flicker (*Colaptes auratus*); common raven (*Corvus corax*) and American crow (*Corvus brachyrhynchos*).

4.5 Conclusion

Colorado is typical of states in the Rocky Mountain region with a growth rate that is three times the national average and manifested in urban and suburban sprawl. These phenomena are particularly acute in counties along Colorado's Front Range, where approximately 80% of the states population lives. There, annual population increases exceeding 4-6% are not uncommon (US Bureau of the Census 1998) however this tendency will in the long run subject urban lakes increasingly more to negative environmental impacts like storm water nutrients and contaminant loading which is synonymous to urbanization. Adding water from Pleasant Valley, Cache la Poudre River and the Colorado-Big Thompson watershed to Sheldon Lake could also be ecologically damaging for it usually brings in water of different chemical nature and may introduce alien organisms. The observed large proportion of collector-gatherers and large fraction of organic pollution-facultative organisms is indicative of excessive organic loading in Sheldon Lake during our survey. Moreover, due to the loss of aquatic vegetation through bottom sediment removal, Sheldon Lake formed a turbid phytoplankton-dominated state instead of a clear macrophyte-dominated state. The submerged vegetation that was lost during the restoration process could have enhance water clarity by reducing resuspension of bottom material, providing zooplankton with a refuge from grazing by planktivorous fish, egg-laying sites and source of food for a wide variety of invertebrates, suppressing algal growth by competing for

nutrients, and releasing allelopathic substances that are toxic to algae (Scheffer 1998). However, the loss of the submerged macrophyte communities in general caused reduction in diversity of keystone macroinvertebrate and zooplankton species that acted as a key in determining much of Sheldon Lake's ecological function.

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CHAPTER 5

Toxic cyanobacterial blooms in a shallow artificially mixed urban lake Colorado

The contents of this chapter have been submitted for publication to *Lakes & Reserviors: Research and Management*.
(Manususcript submission number: 2005/10; Date of acceptance: February 13, 2006)

Abstract

One of the most severe problems related to urban freshwater ecosystem eutrophication is the occurrence of increasingly frequent blooms of toxic cyanobacteria. Cyanotoxins may accumulate in the trophic web and produce diverse intoxication symptoms and chronic effects that are difficult to diagnose and prevent. High mortality of domestic animals and fish has been reported previously under these prevailing conditions. In this study we investigated the taxonomic composition of phytoplankton assemblages during the summer of 2004, a year after restoration of Sheldon Lake. We also analyzed the physical and chemical changes caused by urban runoff, artificial mixing, as well as the usefulness of microcystin molecular markers derived from the *mcy* gene cluster for the detection of toxic cyanobacterial strains in environmental samples of Sheldon Lake. Our study clearly demonstrates that the artificial mixing rate alone is insufficient to cause a transition to a well-mixed system, and that cyanobacteria remained dominant all throughout the summer months. We confirmed the presence of the toxic cyanobacterial strains through the use of molecular markers that detect the presence of the *mcy* gene cluster that is responsible for the production of toxin by *Microcystis* spp. This may have great use-potential in routine analysis of urban aquatic ecosystems. Thus, it may make water monitoring more feasible and allow for the early application of corrective actions especially in cases like Sheldon Lake that is a public recreational focal point.

Keywords: urban runoff, artificial mixing, eutrophication, *Microcystis* spp., molecular markers

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5.1 Introduction

Some of the most serious effects of urban runoff are on the aquatic habitat of the receiving waters. Water quality within large spectrum of urban water bodies like reservoirs for flood control or drinking water, stormwater management ponds, or recreational-park ponds is often under great pressure, owing to impacts such as stormwater nutrient and contaminant loading. Urban runoff may carry large amounts of sediment into a receiving waterbody, reducing water clarity and modifying the benthic substrate. Unfortunately sediment is not the only objectionable substance that is found in urban runoff. Urban runoff may also contain high concentrations of nutrients, oxygen-consuming wastes, pathogens, and toxic substances such as pesticides, heavy metals and oil (Peterson *et al*. 1985). Heaney *et al*. (1980) reviewed fish-kill information reported to government agencies from 1970 to 1979 in the U.S. and found that less than 3% of the reported 10 000 fish kills were identified as having been caused by urban runoff. Especially prevalent within urban water bodies are concerns about the amount and distribution of nuisance-forming cyanobacteria. Cyanobacterial blooms may cause a variety of water quality problems, including dissolved oxygen depletion and subsequent fish kills, aesthetic nuisances (e.g., odours, scum fish tainting, unsightliness) and unpalatable and possibly unsafe drinking water (Carmichael 2001). Such problems can severely limit aquatic habitat, recreation activities, fisheries, and use of a water body as a potable water resource.

Cyanobacteria are known to produce a variety of toxins that can be lethal to livestock, pets, wildlife and humans following ingestion of water contaminated with toxic cells

or toxins released from decaying cells (Codd 1999; Azevedo *et al*. 2002). Certain species of *Anabaena* and *Aphanizomeno*n, for example, synthesize neurotoxic alkaloids, whereas species of *Anabaena, Microcystis*, *Nodularia* and *Planktothrix* mostly generate hepatotoxic peptides (Carmichael *et al*. 1990). The latter secondary metabolites are contained within the cells and are usually only released upon lyses or when changes in cell wall permeability occur. One of the most common genera in cyanobacteria blooms, *Microcystis*, produces the hepatotoxin microcystins which are rarely reported in high concentrations of open lake water, but concentrations of some hundred µg/L are occasionally encountered at near-shore sites where cyanobacteria accumulate. Children are particularly at risk of ingesting major amounts of cyanobacterial cells when playing in the shallow zones along lakeshores where toxic cyanobacterial scum accumulates. Further support for this statement comes from a Swedish investigation in which the levels of toxicity at different depths of a *Microcystis* bloom were determined (Annadottér *et al*. 1991). Annadottér *et al*. (1991) found that the toxicity was highest at the surface water near shore and decreased with depth during periods of calm weather. Thus, recreational exposure to cyanobacteria should be assessed with respect to the possibility of health hazards both through dermal exposure to cyanobacterial cells and their poorly understood irritable and/or allergenic components, as well as through systemic exposure due to accidental ingestion and aspiration of cell material (Chorus & Mur 1999).

Mammals and birds in general appear to be more susceptible to these toxins than are aquatic invertebrates and fish. Death of fish during cyanobacterial blooms has been reported (Davidson 1959; Koon 1960; Ochumba 1990; Sevrin-Reyssac & Pletikosic 1990) although the evidence linking their deaths to toxins is uncertain.

Histopathological investigations of fish deaths during cyanobacterial blooms in the UK indicated that the cause of death was due to damage to the gills, digestive tract and liver (Rodger *et al*. 1994). However, not all species of cyanobacteria synthesize toxins and the potency of blooms can substantially vary between sites, seasons, weeks, or even days. This variation could be due to changes in species composition, production of different toxins with varying toxicity by one clone, and other reasons influenced by environmental factors (Benndorf & Henning 1989). Since the discovery of the *mcy* gene cluster responsible for microcystin biosynthesis in *Microcystis* it was possible to develop DNA primers for PCR assays, which were then used to discriminate between toxic (i.e., microcystin-producing) and non-toxic (non-microcystin-producing) genotypes (Dittmann *et al*. 1997; Nishizawa *et al*. 1999, 2000; Tillett *et al*. 2000, 2001; Kurmayer *et al*. 2002). The aims of the present study were to use the *mcy* gene cluster to detect the toxicity status of *Microcystis* populations in Sheldon Lake. By doing so it will provide us with more information on the microcystin producing genotypes that form the epilimnetic population and help predicting the formation of toxic blooms, as well as monitoring their development in urban lakes. Also, the study aimed to assess the effect of urban runoff on the 'health' of Sheldon Lake, as well as artificial mixing as a management tool to prevent the formation of blooms.

5.2 Study site description and background

Since the early 1900's, City Park has been a favorite recreational spot for Fort Collins residents in Colorado. The 6,07 hectare Sheldon Lake was excavated in 1874, and is a focal point of the 34,39 hectare park (Fig. 5.1). The area around City Park, the

adjacent cemetery and nine-hole golf course, is highly developed with arterial streets, schools, businesses, residential houses and apartments. In the early years, little thought was given to storm drainage as these areas developed. In 1997, 20.32 cm of rain caused a 5 m high railroad bed, acting as a dam, to break, flooding the city. The flood caused five deaths and millions of dollars in damage (Endres 2004). In December 2002, the Sheldon Lake Drainage Improvement Project started by removing over a 100 years of accumulated sediment at the bottom of the lake to increased the water detention with an additional 30 837.225 m^3. The Sheldon Lake Drainage project was completed in June 2003 at a total cost of 6 million dollars. During this time 746.76 m of storm sewer pipe, 457.2 m of box culvert, 274.32 m of water line and nine stormwater inlets were installed, 30 837.225 m^3 of lake bottom sediment were removed and approximately 21 000 fish were relocated. After completion of the project in June 2003 the lake was restock with largemouth bass (*Micropterus salmoides*); bluegill sunfish (*Lepomis macrochirus*); crappie (*Pomoxis annularus*); channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*). To regulate lake depth variation during summer, water is piped from Pleasant Valley, Cache la Poudre River and the Colorado-Big Thompson watersheds (Sheldon Lake Drainage Improvement Project 2002, 2003a,b).

To enhance water quality in Sheldon Lake, artificial mixing was introduce to manipulate the physical environment so that it becomes less favorable for cyanobacteria and more favorable for less problematic species. The lake was mixed artificially with aerators that were placed on the bottom. Compressed air bubbles were passed from the aerators into the water and were visible as surface boils. Two air compressors connected to a network of tubes provide air. Because of the relatively

high floating velocity of the cyanobacterial colonies, the equipment for aeration was designed in such a way that the vertical mixing velocity would be sufficient to keep

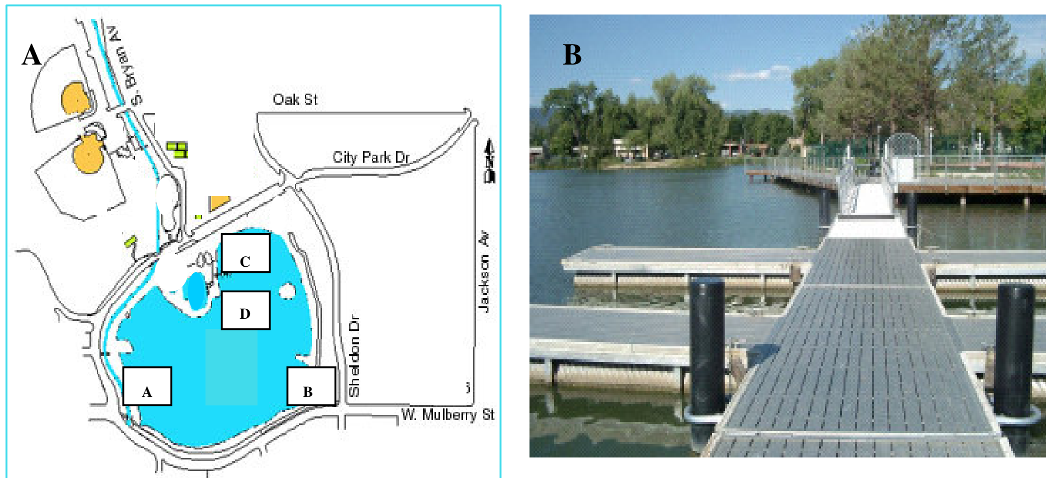

Figure 5.1 (A) Map of Sheldon Lake, Colorado (Sheldon Lake Drainage Improvement Project 2002) (Scale 10 m = 4 mm). (B) Sampling site D.

cyanobacteria entrained in the turbulent flow. In the short term, artificial mixing could prevent cyanobacterial bloom forming since this organism benefits from a stable, stratified water column (Kohler 1992). Gas vesicles provide the cyanobacterial colonies with buoyancy, which enables them to concentrate their biomass in the upper mixed layer and to maximize light interception. In this way, cyanobacteria increases its total daily light dose during periods of calm weather (Ibelings *et al*. 1991; Kohler 1992) while non-buoyant phytoplankton suffers from increased sedimentation losses in a stable lake. In July 2004, a year after the restoration of Sheldon Lake a bloom of *Microcystis wesenbergii* (Teiling 1941; Wojciechowski 1971) started to developed near the shores. This was succeeded by a bloom of *Woronichinia naegeliana* (Smith 1950) in early August, which was precede by a bloom of *Microcystis aeruginosa* (Smith 1950) during the middle of August. The *Microcystis aeruginosa* presented an exponential growth phase that progressed until the middle of September. This phase

was immediately succeeded by a fast declining phase. Beyond this decline *Microcystis aeruginosa* persisted in low numbers and disappeared from the water column at the end of October.

5.3 Material and methods

5.3.1 Bloom Sampling

We sampled the lake two weekly from July to the end of September 2004. The sampling was carried out in the morning (8.00-9.00) and afternoon (16.00-17.00). Four sampling stations were selected. The water column depths of the sampling stations varied from 0.6 to 0.8 m (station A), 0.5 to 0.65 m (station B), 0.4 to 0.5 m (station C) and 0.6 to 0.75 m (station D) due to natural fluctuating intra-and inter-annually depending largely on regional climatic conditions and urban runoff within the sampling area. Water was sampled from the top 0.35 m of the water column, using a Van Dorn bottle. In the laboratory, natural bloom sample water was divided into three sets, the first set were decanted in glass cylinders and placed under fluorescent lights. Under these conditions, the cyanobacteria floated to the surface. The lower water layers were siphoned off, and the cyanobacterial cells were collected, identified and stored at 4 ºC for no more than 1 week, whilst culture was purified for DNA/RNA extrations (Kurmayer *et al*. 2002). Glycerol stocks were also prepared and stored at -80 ºC until further use. Material used for determination of toxicity was processed immediately and frozen at -20 ºC until assayed. The second set of samples was used to determine species composition and community structure. Composition and density were assessed from 1 mL aliquots sampled from 100 mL vertical water samples, then

fixed with buffered 5% (v/v) formaldehyde. Handling, identification and counting of phytoplankton followed the procedures of Lund *et al*. (1958), Brower & Zar (1977), and Padisak (1993). The third set of samples were filtered onto filters with a pore size of 1.0 µm and stored at –20 ºC until chlorophyll*a* (Chl*a*) extraction. For the characterization of water quality during the cyanobacteria bloom period, some physico-chemical and biological variables (temperature, pH, Chl*a*, total nitrogen and phosphate, silica and ammonia, secchi depth, rainfall, windspeed and stormwater runoff) were determined. The entrainment of cyanobacterial colonies in the turbulent flow was investigated by determination of the buoyancy state of the vertical distribution of *Microcystis* colonies, which was measured in the morning and afternoon of 22 August by sampling at intervals of 0.25 m at the different stations. The samples were fixed with 5% (v/v) Lugol's and were counted in a Sedgewick-Rafter chamber.

Chlorophyll*a* (Chl*a*) was measured by the extracting of Chl*a* from lyophilized filters using N,N-dimethylformamide for 2 h at room temperature. Chl*a* was measured spectrophotometrically at 647 and 664 nm and calculated according to Porra *et al.* (1989). Temperature profiles and pH of the water column were measured with an YSI model 2100 thermometer and a 211 microprocessor pH meter. Data of wind speed, direction and rainfall was measured at the meteorological station of the Colorado State University, 2 km away from the lake. Nutrients dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) was analyzed using classical spectrophotometric methods (American Public Health Association, American Water Work Association, and Water Pollution Control Federation 1980).

5.3.2 Bloom toxicity confirmation

5.3.2.1 Pretreatments of environmental samples for whole-cell PCR

For whole-cell PCR cyanobacterial cells were collected from the environmental samples by placing the water sample under fluorescent light in a glass cylinder. The cyanobacterial cells floated under these conditions to the surface and the lower water layers were siphoned off. Before resuspension in distilled water to define volume, the cells were washed one to three times with distilled water and purified by picking colonies (Kurmayer *et al*. 2002). DNA was extracted from an axenic PCC7806 culture strain using DNAzol®-Genomic DNA Isolation reagent following the manufacturers' procedures (Molecular Research Center, Inc., USA).

5.3.2.2 PCR amplification

PCR was performed in a GeneAmp2400 thermocycler (Perkin-Elmer Cetus, Emeryville, Calif., USA). The thermal cycling protocol included an initial denaturation at 94 °C for 2 min, followed by 35 cycles. Each cycle began with 10 s at 93 °C followed by 20 s at the annealing temperature at T_m°C for the specific primer pairs (Table 5.1), and ended with 1 min at 72 °C. When extracted DNA was used, the amplification reactions contained a 10x amplification buffer with 1.5 mm $MgCl_2$, 0.2 mm dNTPs, 20 pmol of each primer and 1 U Taq DNA polymerase, and 3-5 ng purified DNA in a final volume of 50 µL (Dittmann *et al.* 1999, Oberholster 2004, Grobbelaar *et al.* 2004, Grobbellaar 2005). The PCR amplification with whole cells started with 6 µL of crude sample, pretreated subsample with an approximate cell

density of 8 x 10^6 cells/ml, or 0.1µg lyophilized cyanobacterial cells. The sample was added directly to a 20-µL-reaction solution containing bovine serum albumin (0.1 mg/mL) or skim milk (0.1-100 mg/mL, w/v), and a 10x amplification buffer, which contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer, and 0.5 U Taq DNA polymerase (Howitt 1996). The PCR amplifications conditions were identical to those for the samples described above. An extra ramp rate of 3 s/°C was set between the denaturing and annealing steps, when a GeneAmp9600 cycler, instead of GeneAmp2400 was used for PCR amplification. The dosage for the skim milk ranging from 1 to 100 mg/mL was determined to be appropriate based on the results of PCR.

Table 5.1 Oligonucleotides used for RT-PCR and PCR analysis.

Gene region and primer	Sequence	T_m (°C)	Fragment size	Authors
McyA NMT				
MSF	5'-ATCCAGCAGTTGAGCAAGC-3'	59	~1.3 Kb	Tillett *et al.* 2001
MSR	5'-TGCAGATAACTCCGCAGTTG-3'	60		
MSI	5'-GAGAATTAGGGACACCTAT-3'	48		
uma1				
UMF	5'-CCTATCGTCGTATTTGGAGT-3'	54	867 bp	
UMR	5'-AAGGAATGGACACGATAGGC-3'	59		
McyB				
Tox 1P	5'-CGATTGTTACTGATACTCGCC-3'	57.9	~350 bp	Oberholster 2004 Grobbelaar et al. 2004 Grobbelaar 2005
Tox 1M	5'-TAAGCGGGCAGTTCCTGC-3'	58.2		
Tox 3P	5'-GGAGAATCTTTCATGGCAGAC-3'	62.4	~350 bp	
Tox 2M	5'-CCAATCCCTATCTAACACAGTACCTCGG-3'	65.1		
Tox 7P	5'-CCTCAGACAATCAACGGTTAG-3'	53.7	~350 bp	
Tox 3M	5'-CGTGGATAATAGTACGGGTTTC-3'	58.4		
Tox 10Pf	5'-GCCTAATATAGAGCCATTGCC-3'	59.8	~350 bp	
Tox 4Mr	5'-CCAGTGGGTTAATTGAGTCAG-3'	57.9		
McyB				
Tox2+	5'-AGGAACAAGTTGCACAGAATCCGCA-3'	50	~200 bp	Kaebernick *et al.* 2000.
Tox2-	5'-ACTAATCCCTATCTAAACACAGTAACTCA-3'	50		
McyD				
McyDF2	5'-GGTTCGCCTGGTCAAAGTAA-3'	50	~297 bp	
McyDR2	5'-CCTCGCTAAAGAAGGGTTGA-3'	50		
McyB				
FAA	5'-CTATGTTATTTATACATCAGG-3'	40	~580 bp	Neilan *et al.* 1999
RAA	5'-CTCAGCTTAACTTGATTATC-3'			

5.3.2.3 RNA extraction and RT-PCR

Picked colonies were homogenized using a mortar and pestle and liquid nitrogen. RNA extracted using the Qiagen RNAeasy kit (Qiagen Inc., USA) according to the manufacturers' instructions. All equiptment, material and solutions were pretreated with Diethyl pyrocarbonate (DEPC) to ensure that it is RNase free. First strand cDNA was synthesised from RNA using the cDNA synthesis System according to the manufacturers' instructions (Roche Molecular Biochemicals, Germany).

Real-time PCR was performed using first strand cDNA (70 ng) from selected total RNA as required. The 20 µL reaction contained 10 pmol forward and reverse primers (Table 5.1), 3 mM $MgCl_2$ and the LightCycler–FastStart DNA Master SYBR Green 1 Mix (Roche Diagnostics Corporation, Germany) as according to manufacturer's procedures (LightCycler–FastStart DNA Master SYBR Green 1 Manual, Roche Applied Science, Germany). Amplification of products consisted of 1 cycle at 95 °C for 10 min; therafter 40 cycles were conducted starting with 1 cycle at 95 °C for 10s, primer specific annealing T°C for 5s, 72 °C for 10s; followed by the melting curve analysis (95 °C for 0s, 65 °C for 15s, 95 °C for 0s), and cooling (40 °C for 30s). A minimum of 7 reactions was done for each fragment analyzed, standard curves were generated using dilution series (1:1, 1:10, 1:100, 1:1 000) and repeated. Results obtained were analyzed using LightCycler Software version 3.5 (Roche Applied Science, Germany) and were expressed as fold change.

5.3.2.4 Fish bioassay

Hepatocytes were isolated from rainbow trout (*Oncorhynchus mykiss*) by a two-step collagenase perfusion of the liver according to Seglen (1976). During the perfusion, buffers were supplemented with 0.2-1% bovime serum albumin. All cells were carried out at 2 x 10^6 cells/ml in a buffer containing 30 mM Hepes, 30 mM TES, 30 mM Tricine, pH 7.4, 68 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1.1 mM KH_2PO_4, 0.7 mM Na_2SO_4 and glucose.

5.3.2.5 Light microscopy

Hepatocytes were fixed for light microscopy in 3% (m/v) paraformaldehyde in phosphate buffer saline (pH 7.4) and mounted on a glass cover slip before viewing using a Zeiss microscope using interference contrast optics.

5.3.2.6 Protein Phosphatase Inhibition and ELISA Assays

Intracellular microcystin content was measured using the colorimetric PP2A inhibition assay (An & Carmichael 1994). The recombinant catalytic subunit of protein phosphatase 1 as expressed in *Escherichia coli* was used as the source of PP enzyme (Zhang *et al*. 1992). This assay has a 50% inhibitory concentration of 6.72 µg/L for the microcystin-LR standard, using 0.5 mg of PP2A per mL, and a limit of detection of 0.033 µg/L or 33 Pm. Prior to the assay, 1 mL of cell suspension in water (supernatant removed) was freeze-thawed three times and diluted 1: 100 to 1: 8 000. Samples were measured in duplicate in the same assay and also in repeated assays. After adjusting each assay to percentages of protein phosphatase activities, the concentration of microcystin was calculated from an average standard curve of all

assays. The PP2A inhibitor in this case microcystin content was calculated for cells per milliliter of sample, resulting in values reported as picomoles of microcystin toxin per cell.

The ELISA assay was conducted with a Quanti TM Kit for microcystins (EnviroLogix, USA). The microcystin concentration was determined from the standard curve of microcystin-LR (Ueno *et al*., 1996). The results were obtained by reading the plate on a multiskan ascent (Thermo Labsystems).

5.3.2.7 Data analyses

The results were recorded on standard Excel spreadsheats for data processing, and statistical analysis was performed using SYSTAT® 7.0.1 (1997).

5.4 Results

5.4.1 Species composition and Physical/chemical measurements

During the study period June to October 2004, cyanobacterial colonies were found on each sampling date, with maximum abundance on 22 August (1.66×10^9 cells/ml) and minimum abundance on 30 June (1.30×10^8 cells/ml) (Fig. 5.2).

Identification of individual cyanobacteria colonies from June to September revealed a complex pattern of cyanobacterial species succession. The occurrence of three

morphospecies: *Microcystis aeruginosa*; *Woronichinia naegeliana* and *Microcystis wessenbergii* were observed during this time. The phytoplankton composition from

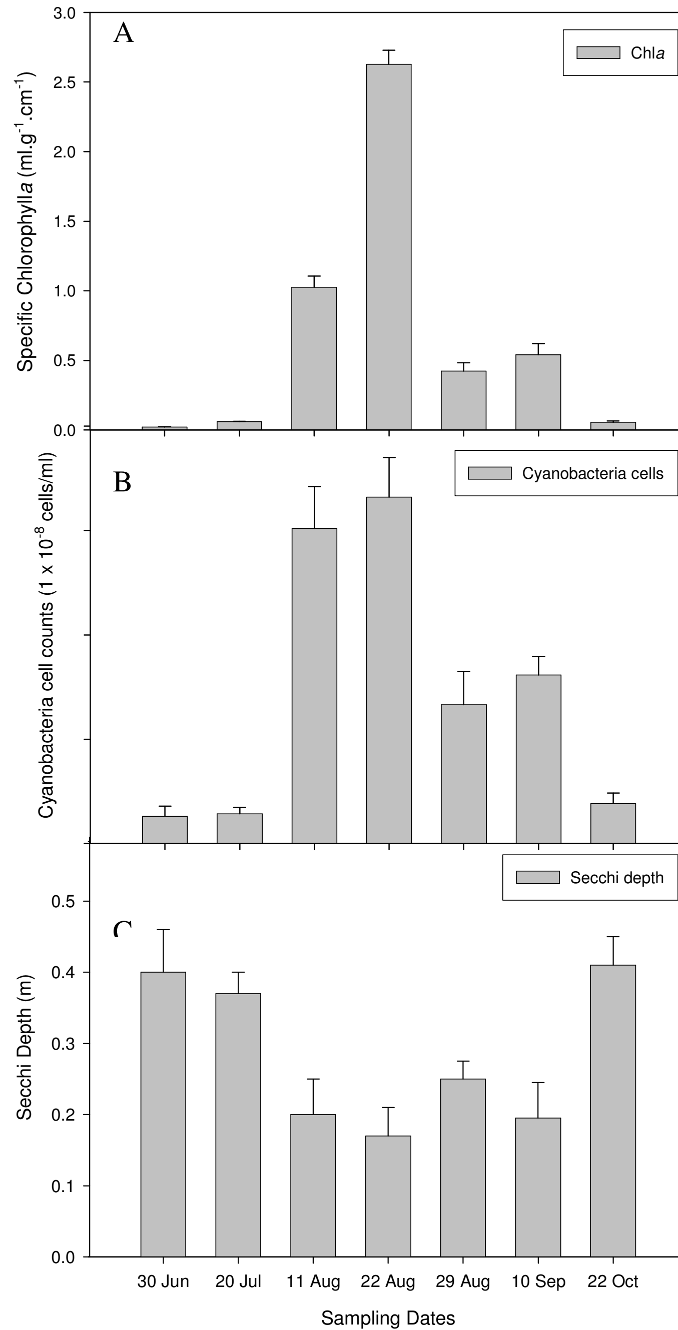

Figure 5.2 Average (a) specfic Chl*a*, (b) cell count and (c) transparency (Secchi depth) during surface blooms in the summer 2004 with artificial mixing, Sheldon Lake, Colorado. Error bars represent standard deviation from the mean value.

live samples taken on three sampling dates in August showed a dominance of Cyanophyceae (*Microcystis aeruginosa*, *Woronichinia naegeliana*) (Fig. 5.3) followed by less dominance of Chlorophyceae (*Cosmarium montrealense*, *Closterium aciculare*); Bacillariophyceae (*Synedra rumpens var fusa*, *Asterionella formosa, Melosira varians*); Euglenophyceae (*Euglena caudate*, *Phacus pleuronectes*) and Chrysophyceae (*Chrysococcus minutus*) (Fig. 5.4).

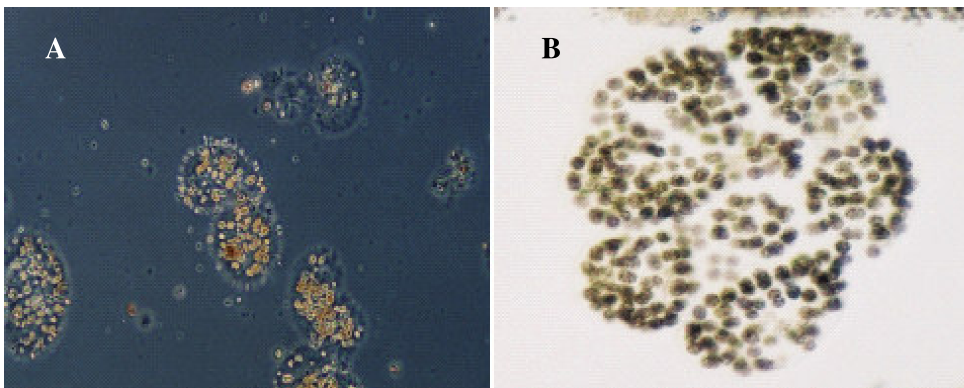

Figure 5.3 (A) *Woronichinia naegeliana* (after Smith 1950); (B) *Microcystis aeruginosa* (after Smith 1950); Unstained, bright-field microscopy, 200 x.

The Secchi depths during the three sampling dates in August were generally restricted to the upper 25 cm of the water column, while a chlorophyll increase together with a 50% reduction in light penetration (as Secchi depth) was observed on 22nd of August 2004 (Fig. 5.2).

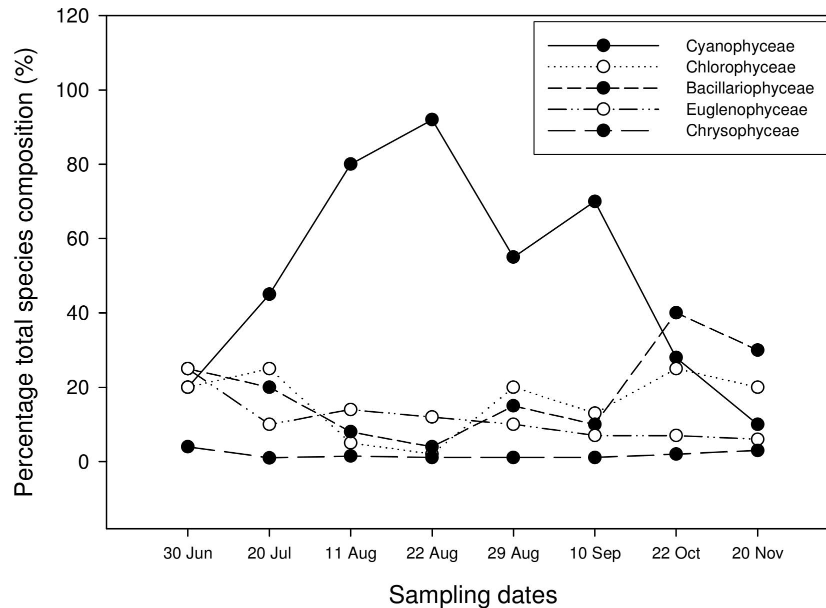

Figure 5.4 Seasonal variations in the relative abundance of five phytoplankton groups with artificial mixing during the summer 2004, Sheldon Lake, Colorado.

Following the Carlson's trophic state index and its associated parameters (Carlson 1977; Kratzer & Brezonik 1981) we presently considered Sheldon Lake eutrophic a year after restoration, due to the summer results of total Secchi disc depth (0.25 m), specific Chl*a* (0.693 mg/L) and Phosphates concentration which ranging from 0.2 to nearly 0.3 mg/L while total Nitrogen value ranging from 0.2 to 0.4 mg/L at the different sampling locations (Fig. 5.2). The concentrations of dissolved inorganic Nitrogen and Phosphates were low while total Ammonia concentration was 0.467 mg/L; Silica 6.3 mg/L and pH 8.9 (also see Chapter 4, Table 4.3).

5.4.2 Artificial destratification

In the morning when samples were taken before artificial mixing of the lake started, an overnight cyanobacteria surface bloom formation was frequently observed, near the shores (Fig. 5.5).

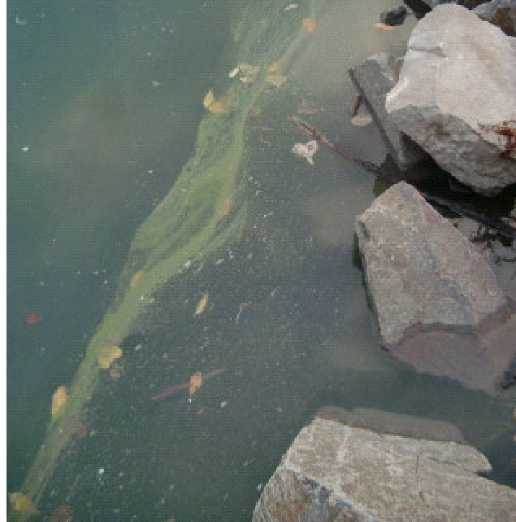

Figure 5.5 Cyanobacterial bloom still visible in the morning on the surface water during artificial mixing at sampling site B.

The average measured wind velocity for most of September was 1.07 ± 0.6 m/s, while the wind speeds increase to an average speed of 10.2 ± 3 m/s during the high-wind and storm events of 4, 21, 25 and 27th September 2004 (Table 5.2).

Table 5.2 Average wind velocity (m/s).

	Year	
Month	**2001**	**2004-2005**
April	0.98	1.02
May	1.04	1.01
June	1.01	1.04
July	1.09	1.07
August	1.13	1.20
September	1.35	1.98
October	1.23	1.34

However during the afternoon when the second set of samples was taken the *Microcystis* bloom next to the shores of the lake had disappeared and a low

concentration of *Microcystis* colonies was found on the surfacewater. We also observed a difference in the number of cyanobacterial colonies with artifical mixing in the morning and afternoon surfacewater at the four sampling sites (Fig. 5.6).

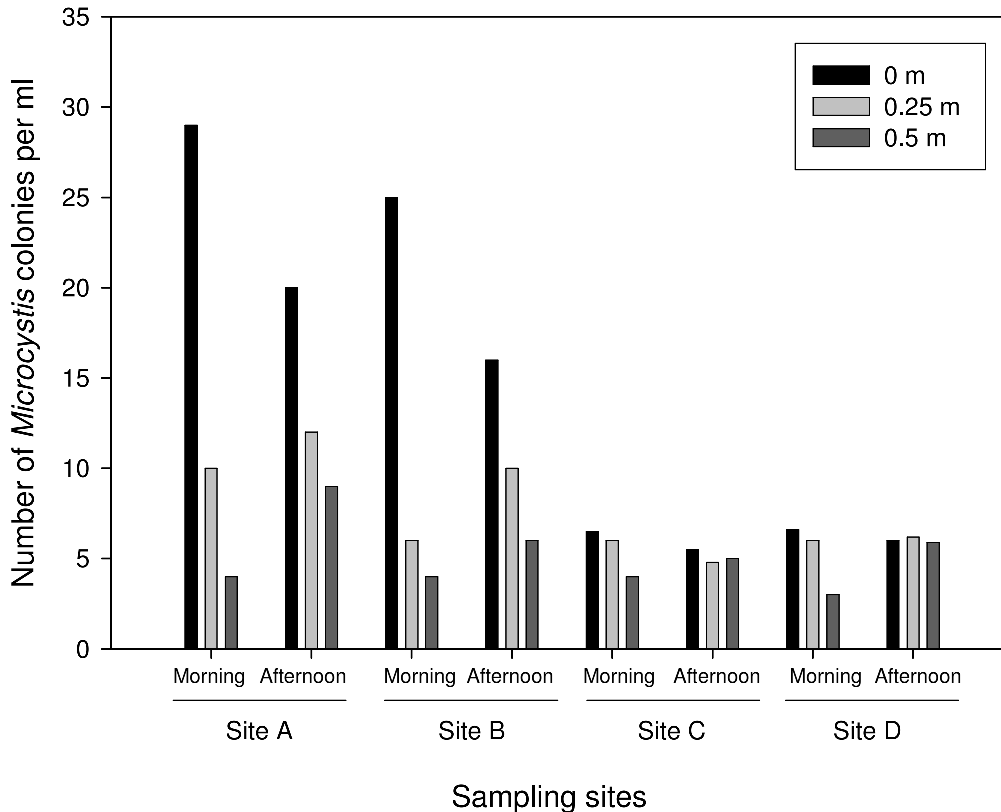

Figure 5.6 The number of *Microcystis* colonies/ml of samples taken at 0.25-m depth intervals at different locations in the lake during artificial mixing, taken on the morning and afternoon of August 22, 2004.

5.4.3 Detection of toxicity

The toxicity of the environment strains were also determined using ELISA and through inhibition of PPA2, and the toxicity levels were comparable to the toxin levels present in the cultured PCC7806 strain (Table 5.3).

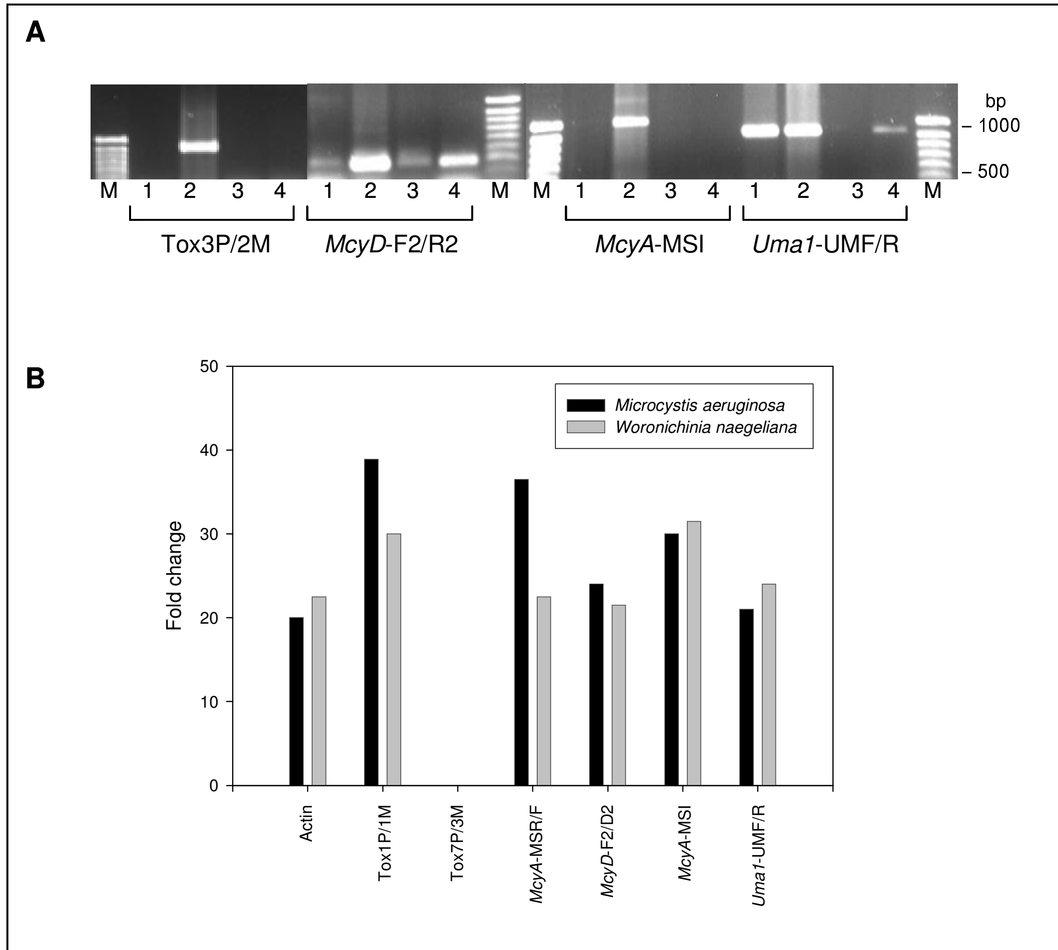

Figure 5.7 (A) Separation of PCR amplicons obtained after PCR of *Microcystis aeruginosa* strain UPUS1 (1); PCC7806 (2); UP37 (3) and *Woronichinia naegeliana* strain UPUS2 (4) using different primers on a 2% agarose gel. M = Hyperladder^{TM} IV, Bioline, USA. (B) Quantitative PCR of RNA from *Microcystis aeruginosa* strains UPUS1 and *Woronichinia naegeliana* strain UPUS2 with selected primers. Actin was included as standard.

Amplification products obtained from the cyanobacteria spp. sampled in Sheldon Lake provided for supporting evidence that the environment *Microcystis aeruginosa* (UP37, UPUS1) and *Woronichinia naegeliana* (UPUS2) strains contained the *mcy* genes present in the culture *M. aeruginosa* strain PCC7806, and that is normally associated with toxin production (Fig. 5.7, Table 5.3). These included amplification products after amplification with TOX1P/1M (*mcyB*) (~350 bp; UP37, UPUS1 and

UPUS2), TOX7P/3M (*mcyB*) (~350 bp; UP37); *mcyA*-MSR/MSF (~1.3 Kb; UPUS1 and UPUS2), *mcyD*-F2/R2 (~297 bp; UP37, UPUS1 and UPUS2), and *Uma1*-UMF/R (~867 bp; UPUS1 and UPUS2) (Fig. 5.7A).

The data was further supported by the expression of selected genes in the *mcy* cluster (i.e., TOX1P/1M, *mcyA*-MSR/MSF, *mcyD*-F2/R2, and *Uma1*-UMF/R) using quantitative PCR on RNA isolated from the environment strains *Microcystis aeruginosa* (UPUS1) and *Woronichinia naegeliana* (UPUS2) (Fig. 5.7B). Similar to the PCR analysis, no expression was obtained after RT-PCR of RNA from the environment strains *Microcystis aeruginosa* (UPUS1) and *Woronichinia naegeliana* (UPUS2) using primer set TOX7P/3M (Fig. 5.7B).

Examination of the rainbow trouts revealed damage to the fins and outer surfaces of the body (Fig. 5.8). Damage to the gills of some of the fish was also observed. Microscopic assessment of the fish hepatocytes revealed clustered blebs and deformation of the cell structure (data not shown).

Figure 5.8 Histopathological investigation of *Oncorhynchus mykiss* revealed damage to gills and fins during cyanobacterial surface blooms during August, 2004.

Table 5.3 Comparison of PCR with different primers, quantitative PCR, ELISA and Protein Phosphatase inhibition (PP2A) assay as determinants of toxicity in strains from different geographical regions. (+ = positive/product; - = negative/no product; / not assayed).

Organism	Isolation date	Geographic origin	PCR										RT-PCR [a]	PP2A* (%)	ELISA (µg/L)
			Tox 3P/2M	Tox1 P/1M	Tox7 P/3M	Tox1 0P/4M	*McyB* Tox2 +/-	*McyB* 2-FAA/RAA	*McyA* -MSR/MSF	*Mcy* *D*-F2/R2	*McyA* -MSI	*Uma1* -UMF/R			
Microcystis aeruginosa:															
PCC7806 (Cultured strain)	1972	Braakman Reservoir, The Netherlands	+	+	+	+	+	+	+	+	+	+	/	100	44.1 ± 31
UP37 (Environment strain)	2004	Krugersdrift Reservoir, ZA		+	+					+			/	100	61.2± 54
UPUS1 (Environment strain)	2004	Sheldon Lake Colorado, US		+					+	+		+	+	100	32.8 ± 15
Woronichinia naegeliana:															
UPUS2 (Environment strain)	2004	Sheldon Lake Colorado, US		+					+	+		+	+	45	5.6 ± 1.6

* % of inhibition effect of mycrocystin-LR on protein phosphatase activity. Inhibition was calculated from the activity of PP2A on a colorimetric substrate. The control used to plot a standard curve was MCYST-LR and activity values between 0% and 100% correspond to the log of inhibitor concentrations of 0-3000 nM (assuming the sole PP2A inhibitors in *M.aeruginosa* to be microcystins and the molecular mass of microcystin-LR is 995.2).

[a] Results obtained with primers used for RT-PCR correspond in all experiments to that obtained with PCR, results were comparable with regard to presence of amplicon.

5.5 Discussion

The phenomenon that *Microcystis* form overnight surface blooms are probably due to the fact that under low light conditions (early morning) cyanobacteria increase their buoyancy or at night, cyanobacteria do not actively regulate their buoyancy (Pearson 1990). Thus, if the artificial mixing stopped overnight they may float to the surface, but because it is dark they cannot reverse their buoyancy. Typically, with an underwater circulating aerator there is considerable turbulence close to the surface boil, but further away it decreases. The consequence of this phenomenon is that the *Microcystis* colonies are entrained close to the surface boil but remained disentrained away from the surface boil. This was demonstrated by Visser *et al*. (1996) where *Microcystis aeruginosa* colonies close to the surface boil of the underwater circulating aerator remained buoyant as they experienced a low mean photon density due to regular mixing, and consequently accumulated little carbohydrate. By contrast, *Microcystis* colonies away from the surface boil of the underwater circulating aerator were not entrained, remained close to the water surface and experienced a higher photon density that caused the cells to accumulate carbohydrate, increase their net cell density and lose buoyancy and sink (Ibelings *et al*. 1991). This phenomenon which indicates differences in buoyancy between locations where horizontal mixing of water masses were restricted to an extent that allowed differences in vertical mixing was confirmed by analyzing the distribution of *Microcystis* colonies with depth during sampling in the morning and afternoon of 22 August 2004. Our data clearly highlighted that sampling sites A and B which were a larger distance from the aerators, had a higher number of sinking *Microcystis* colonies per mL in the afternoon. While sampling sites C and D which are near two surface boils of

underwater aerators show a very low percentage of sinking colonies although site C was much shallower than all the other sampling sites (Fig. 5.6). Out of our data we determined that artificial mixing does not prevent bloom forming of cyanobacteria at sampling sites A and B and that the mixing in those areas of the lake was insufficient at an average wind speed of 1.2 m/s.

Kortmann *et al.* (1994) reported that aeration is not always successful in reducing the high relative abundance of the large scum-forming and odour-producing cyanobacterial species or concentrations of available phosphorus. In fact, transparency declined following artificial circulation in over 50% of reported case studies.

5.5.1 Incomplete mixing

In our field studies we observed that the artificial mixing rates in Sheldon Lake alone are insufficient to cause a transition to a well-mixed system and therefore the same species cyanobacteria (i.e., *Microcystis aeruginosa*) remained dominant throughout summer (Fig 5.6). During the end of September major changes in species composition happened when there was a true transition from an incomplete to a complete mixing due to high-wind and storm events the 4, 21, 25 and 27th of September. The average recorded rainfall during the month of September was 7.42 cm. The average windspeed measured for September was 1.07 ± 0.6 m/s, while the wind speeds increase to an average speed of 10.2 ± 3 m/s during the above high-wind and storm events (Table 5.2). Since Sheldon Lake is also a recreational focus-point in Ft. Collins, the lake is surrounded by many large tree species, i.e., poplar, and coniferous species, sheltering the lake against prevailing winds. General ecological theory predicts that incomplete

mixing should promote species coexistence (Levin 1974; Atkinson & Shorrocks 1981; Powell & Richerson 1985; Hsu & Waltman 1993; Tilman 1994).

Field studies of Jacobsen and Simon (1993) on a shallow eutrophic lake in Denmark support these predictions. They observed a massive summer bloom of the cyanobacterium *Aphanizomenon* flos-*aquae*, which at the end of July, during a period of high winds and rainfall collapsed, from a biomass of 140 mm^3/L in July to 6.2 mm^3/L in early August. The lake was still stratified, though, and *Aphanizomenon* continued to dominate, although at much lower biomass. In late August, new storms destroyed the stratification, and *Aphanizomenon* was replaced by cryptomonad species.

The increase in diatom standing stocks in the surface waters (0.25 m) of Sheldon Lake during September was associated with a higher average daily wind speed. Their inoculation into the water column under periods of high wind can lead to significant changes in the phytoplankton composition (Carrick *et al*. 1993). Based on the observations of Smetacek (1985) sedimentation of diatoms can be an integral part of their life cycle and enhance survivorship by incidental predator avoidance or redistribution to more nutrient-rich waters. Padisak *et al*. (1988) stated that as wind turbulence increased, the structure of the surface-water community is altered by the increase in algal biomass due to the results from resuspension of diatoms. Because these alterations occur within brief time frames (hours to days) depending of the meteorological activities (wind velocity and rain), we observe that these processes can account for some of the temporal variation in phytoplankton in Sheldon Lake and thus enhances temporal heterogeneity during October.

5.5.2 Toxicity

The morphological discrimination between toxic and nontoxic cyanobacterial strains are not possible as some cyanobacteria are known to be toxic, some may be genetically capable of producing toxins but do not under all conditions, and some do not produce toxins at all. Since the recently discovery of the *mcy* genes coding for subunits of the microcystin synthetase in *Microcystis* (Dittmann *et al*. 1997; Nishizawa *et al*. 1999, 2000; Tillett *et al*. 2000) it is possible for the first time to develop DNA primers for simple PCR assays, which were then used to identify strains bearing *mcy* genes. By using the PCR assays it could be demonstrated that the occurrence of *mcy* genes in cells is correlated with their ability to synthesize microcystin and vice versa (Kurmayer *et al*. 2002).

In most environmental samples that was analyzed the relative intensity of *mcyB* and *mcyD* bands are not approximately equal. The differential amplification of environmental samples may be indicative of the presence of both nontoxic and toxic *Microcystis* (Ouellette & Wilhelm 2003). In our study, we measured toxicity in the cultured strain *Microcystis aeruginosa* (PCC7806) and environmental strains of *M. aeruginosa* (UP37, UPUS1), as well as in an environmental strain of *Woronichinia naegeliana* (UPUS2) using protein inhibition and ELISA assay. We further observed the expression of selected genes in the *mcy* gene cluster after quantitative PCR of RNA isolated from *M. aeruginosa* (UPUS1) and *Woronichinia naegeliana* (UPUS2), although to our knowledge the latter was not reported previously as toxic. The only cyanobacterial colonies observed by microscopic observation during the surface

bloom of 11th August 2004 was *Woronichinia naegeliana*, while the bloom of 22nd August was dominated by *M. aeruginosa* (Fig. 5.3).

In urban eutrophic aquatic systems like Sheldon Lake, fish stand at the top of the aquatic food chain, and are possibly affected by exposure to toxic cyanobacteria. Cyanotoxins may accumulate in the trophic web (Amorim & Vasconcelos 1999; Saker & Eaglesham 1999) and produce diverse intoxication symptoms and chronic effects that are difficult to diagnose and prevent (Falconer 2001). Many experimental studies have been conducted to document the toxicity of microcystin exposure though gastrointestinal or blood systems on fish such as common carp (Råberg *et al*. 1991; Fischer *et al*. 2000; Li *et al*. 2001; Wu *et al*. 2002), rainbow trout (Sahin *et al*. 1996; Bury *et al*. 1996, 1997; Tencala & Dietrich 1997; Fischer *et al*. 2000) and channel catfish (Zimba *et al*. 2001). However, all these studies were limited to acute toxic experiments, and they were based on either oral gavaging, or intraperitoneal injection, or administration via the dorsal aorta of the toxins, which cannot reflect the uptake route under natural conditions.

Toxins used in these experiments were dried cyanobacteria containing microcystin or purified microcystin, which do not evaluated long-term exposure in natural environments. Histopathological investigation done by us on some rainbow trout deaths during cyanobacteria blooms in Sheldon Lake indicated that the cause of death was likely to the result of damage to the gills and fins (Fig. 5.8). Gill damage by dissolved microcystin-LR has been shown experimentally in tilapia and trout (Garcia 1989; Gaete *et al*. 1994; Bury *et al*. 1996). However, gill damage may also be caused by the high pH values associated with cyanobacterial photosynthesis and the high

ammonia concentrations arising from the decomposition of cyanobacterial cells. Irrespective of this, gill damage almost certainly enhances microcystin uptake leading to liver necrosis. Nevertheless it should be pointed out that histopathological investigation was done on already dead rainbow trout, collected near the shores of Sheldon lake, and that there was no control of the same age to compare our results with.

5.6 Conclusion

This study has shown that Sheldon Lake, during the summer of 2004 a year after restoration, contained toxigenic strains of cyanobacteria and are subject to increasingly negative impacts such as stormwater nutrients and contaminant loading which lead to eutrophication. Especially prevalent within Sheldon Lake, which is a prime fishing spot for residents of Fort Collins, is the concern of health hazard for people and domestic animals from cyanotoxins. Although intoxication has not yet been clearly demonstrated, bioaccumulation of four different cyanotoxins in the trophic chain was observed (Falconer *et al*. 1992; Lindholm *et al*. 1989; Saker *et al*. 1999). However due to the fact that research did not yet clarify long-term effects of cyanotoxins, people should limit or avoid eating fish caught there. Furthermore, we find the PCR assays that were applied during the study of cyanobacterial strains, sensitive in providing a useful indicator of toxicity, although it does not provide a direct measure of the toxin present, it detect toxigenic cells rather than toxins and require little sample preparation and modest capital cost.

Acknowledgements

The authors would like to express their sincere gratitude the National Foundation of Research, and the Water Research Commission, South Africa for provision of funding.

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CHAPTER 6

Assessment of the genetic diversity of geographically unrelated *Microcystis aeruginosa* strains using Amplified fragment length polymorphisms (AFLPs)

The contents of this chapter were published in *African Journal of Biotechnology* 4, 389-399, May 2005.

Abstract

Molecular marker analysis is becoming increasingly capable of identifying informative genetic variation. Amplified fragment length polymorphism markers (AFLPs) are among the recent innovations in genetic marker technologies, and provide a greater capacity for genome coverage and more reproducible results than previous technologies. We have investigated the usefulness of AFLP, which is based on the selective amplification of genomic restriction fragments by PCR, to differentiate between geographically unrelated *Microcystis* strains. In total 23 strains were subjected to the AFLP fingerprinting. After analysis of the data on the basis of the average linkage method, known as the Unweighted Pair Group Method using Arithmetic averages (UPGMA), a dendrogram with four clusters was obtained. Cluster 1 consisted mainly of the NIES strains that originated from Japan, while in cluster 2 the European strains grouped together. The South African strains that originated from the northern part of the country group together in cluster 3, while the strains collected from the central and southern regions group together with the US strains in cluster 4. The study had reveals extensive evidence for the applicability of AFLP in population studies, and furthermore clearly demonstrates the superior discriminative power of AFLP towards the differentiation of geographical unrelated *Microcystis aeruginosa* strains that belong to the same species.

Keywords: Molecular markers, distribution, cyanobacteria, genetic diversity**.**

6.1 Introduction

The phylum Cyanobacteria is large and diverse, containing over 1000 species of oxyphototrophs, and its members are classified by using both botanical and bacteriological taxonomic codes (Castenholz *et al.* 1989; Komárek 1991a, b; Komárek & Anagnostidis 1986, 1989; Rippka 1988; Rippka *et al.* 1979). The taxonomy and classification of cyanobacteria has been under investigation since about the middle of the 19th century using morphological and cellular criteria, similar to other microalgae (Kützing 1849; Nägeli 1849 as cited by Komárek 2003). Because of morphological simplicity of most prokaryotes, their classification was previously based largely on physiological properties, as expressed in pure laboratory cultures (Doers & Parker 1988). While field studies relied mostly on morphological analyses of natural populations, laboratory studies concentrated on culture characterisations. The principle of morphological studies includes the use of characteristics observable and measurable under a light microscope, such as shape of colony, presence of sheaths and envelopes, colour of colonies, differentiation and cell content. Based on these criteria, the traditional taxonomic classification systems of cyanophytes placed a high value on cell division patterns, colony formation and relationship to extracellular envelopes and sheaths. Cell shapes and dimensional differences were used largely to distinguish between species within each genus (Doers & Parker 1988). This method caused difficulties in their classification by introducing organisms with different cell organizations but similar cell arrangements to the same generic identity. Furthermore, it required considerable expertise to identify species since both morphological and developmental characteristics can vary with the growth conditions (Evans *et al*. 1976). The main problems met in applying morphological criteria in cyanophyte classification arise from the considerable variability in morphological features with indifferent environmental conditions (Komárek 1991a, b).

Diversification through ecological acclimation, adaptation and stabilization of diverse morpho- and ecotypes, as well as changes caused by mutation and possibly also genome transfers (Rudi *et al*. 1998) can give rise to new cyanobacterial types in different geographical locations. Nearly all populations of cyanobacteria from different geographical locations differ to some degree from each other, and these deviations may stabilize in long term cultures (Waterbury & Rippka 1989; Kato & Watanabe 1993). This process indicates that new forms continually develop and are stabilized under new constant conditions. Diversification within the cyanobacteria is a continuing process in which new types develop from continually modified cyanobacterial genotypes under different environmental conditions at different geographical locations. Many investigations showed that blooms of *Microcystis* spp. can differ in important characteristics (Cronberg & Komárek 1994). The cyanobacteria *Microcystis aeruginosa* is characterized by the existence of a wide variety of genotypes that differ in their content of secondary metabolites (Martin *et al*. 1993; Moore 1996), plasmid content (Kohl *et al*. 1988; Schwabe *et al*. 1988) and interaction with zooplankton (Hanazato 1996). Recent data also reveal variations within cyanobacterial genomes, such as changes in the toxicity of certain strains (Neilan *et al*. 1997) and possible interchanges of genetic material (Rudi *et al*. 1998). Other well-adapted phytoplankton forms, so called traditional species like *Aphanothece, Chroococcus* and *Hyella* appears to persist over the longterm and contain a wide spectrum of stable types for long periods (Komárek & Anagnostidis 1998). Due to the development of recent molecular techniques new approaches have been introduced to the phylogeny and taxonomy of cyanobacteria. Several methods of molecular biology have to date been successfully applied in aid of cyanobacterial

taxonomy: determinations of DNA base ratios (Herdman *et al*. 1979a, b), DNA-DNA hybridisations (Stam 1980; Wilmotte & Stam 1984; Stam *et al*. 1985) and gene sequencing (Masui *et al*. 1988; Nielan *et al.* 1997; Lyra *et al*. 2001; Gugger *et al*. 2002).

The use of DNA-based genetic markers (Bodstein *et al*. 1980) has forever changed the practice of genetics. Over the past 20 years since that discovery, many different types of DNA-based genetic markers have been used for the analysis of genetic diversity, as well as for applied diagnostic purposes (Powell *et al*. 1996). The use of modern molecular biological techniques to determine the degree of sequence conservation between bacterial genomes has led to the development of methods based solely on the detection of naturally occurring DNA polymorphisms. These polymorphisms are a result of point mutations or rearrangements (i.e., insertions and deletions) in the DNA and can be detected by scoring band presence versus absence in banding patterns that are generated by restriction enzyme digestion and DNA amplification procedures. The underlying idea is that variation in banding patterns are a direct reflection of the genetic relationship between the bacterial strains examined and therefore, that these banding patterns can be considered as genomic fingerprints allowing numerical analysis for characterization and identification. AFLP markers have been used to scan genome-wide variations of strains, or closely related species, that have been impossible to resolve with morphological features or other molecular systematic characters. Therefore, AFLP has broad taxonomic applicability and have been used effectively in a variety of taxa including bacteria (Huys *et al*. 1996).

AFLP analysis is based on selective amplification of DNA restriction fragments (Vos *et al*. 1995). It is technically similar to restriction fragment length polymorphism analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. This prevents over interpretation or misinterpretation due to point mutations or single-locus recombination, which may affect other genotypic characteristics. The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer *et al*. 1998). PCR has proven to be successful in detecting genetic variation amongst plant-pathogenic fungi, as well as bacteria (Majer *et al*. 1996; Janssen *et al*. 1996). The utility, repeatability and efficiency of the AFLP technique are leading to broader application of this technique in the analysis of cyanobacteria populations (Janssen *et al*. 1996). The purpose of this study was to investigate the usefulness of AFLP, which is based on the selective amplification of genomic restriction fragments by PCR, to differentiate between geographical unrelated strains of *Microcystis* spp.

6.2 Material and Methods

6.2.1 Chemicals, Strains and Culture Conditions

Analytical reagent grade chemicals were purchased from various commercial sources and were used without further purification. *Microcystis aeruginosa* strains used in the study represented a wide variety of geographically unrelated strains (Table 6.1). Strains PCC7806 and PCC7813 were obtained from the Pasteur Institute Culture Collection, France; UV027 from the University of the Free State Culture Collection, South Africa; CCAP1450/1 was obtained from the Culture Collection of Algae and

Protozoa, Institute of Freshwater Ecology, UK; NIES88, NIES89, NIES91, NIES99 and NIES299 from the National Institute for Environmental Studies, Japan; and SAG1 from the Pflanzen Physiologisches Institut, Universität Gottingen, Germany. All these strains were received as axenic, maintained as such and microscopically verified prior to further experiments.

Table 6.1 Different strains used in the study and their origin.

Strain	Source	Origin
Microcystis spp.		
M. aeruginosa strain PCC7806	Pasteur Culture Collection, France	The Netherlands
M. aeruginosa strain PCC7813	Pasteur Culture Collection, France	Scotland
M. aeruginosa strain UV027	University of the Free State Culture Collection	Germany
M. aeruginosa strain NIES88	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES89	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES91	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES99	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES299	National Institute for Environmental Studies	Japan
M. aeruginosa strain SAG1	Pflanzen Physiologisches Institut, Universität Gottingen	Germany
M. aeruginosa strain CCAP1450/1	Institute of Freshwater Ecology	UK
M. aeruginosa strain UP01	University of Pretoria Culture Collection	Rietvlei, ZA
M. aeruginosa strain UP04	University of Pretoria Culture Collection	Hartbeestpoort, ZA
M. aeruginosa strain UP06	University of Pretoria Culture Collection	Paardekraal, ZA
M. aeruginosa strain UP09	University of Pretoria Culture Collection	Hartbeespoort, ZA
M. aeruginosa strain UP10	University of Pretoria Culture Collection	Roodeplaat, ZA
M. aeruginosa strain UP15	University of Pretoria Culture Collection	Bon Accord, ZA
M. aeruginosa strain UP37	University of Pretoria Culture Collection	Krugersdrift, ZA
M. aeruginosa strain UP38	University of Pretoria Culture Collection	Hartbeespoort, ZA
M. aeruginosa strain UPUS1	University of Pretoria Culture Collection	Ft Collins USA
M. wesenbergii strain UP02	University of Pretoria Culture Collection	Rietvlei, ZA
Other genera		
Chroococcidiopsis cubana strain UP13	University of Pretoria Culture Collection	Klipvoor, ZA
Woronichinia naegeliana strain UPUS2	University of Pretoria Culture Collection	Ft Collins USA
Scenedesmus acutus strain UP26	University of Pretoria Culture Collection	Klipvoor, ZA

Unicellular strains UP01, UP03, UP04, UP10, UP13, UP26, UP37 and UP38 were collected by the authors, representatives of the Water Research Commission and Tswhane Metro Council, respectively. The UPUS1 and UPUS2 were collected at Sheldon Lake, Colorado by the authors. Outgroups included in the study were the

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division Cyanophyta: UPUS2 *Woronichinia naegeliana* (Smith, 1950); UP03 *Microcystis wessenbergii* (Teiling 1941; Wojciechowski 1971); UP13 *Chroococcidiopsis cubana* (Komárek & Hindák 1975) (Fig.6.1) and the division Chlorophyta: UP26 *Scenedesmus acutus* (Ettl & Gärtner 1995).

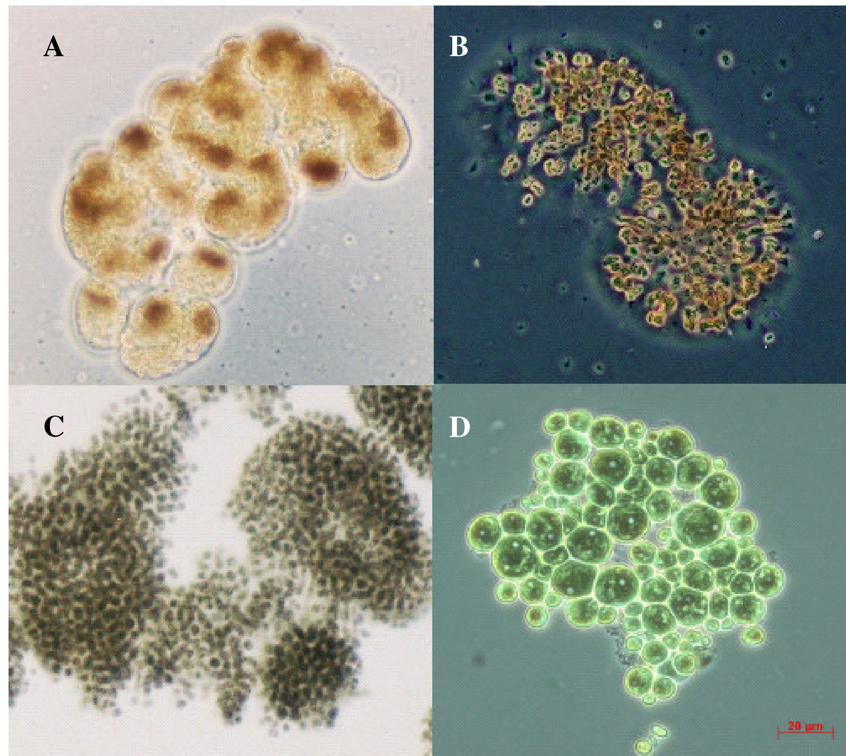

Figure 6.1 (A) *Microcystis wessenbergii* (after Teiling 1941, Wojciechowski 1971); (B) *Woronichinia naegeliana* (after Smith 1950); (C) *Microcystis aeruginosa* (after Smith 1950); and (D) *Chroococcidiopsis cubana* (after Komárek & Hindák 1975). Unstained, bright-field microscopy, 200 x.

After collecting the water samples were placed on ice in a darkened cooler during transport to the laboratory. Holding time for samples was less than 48 h in all cases. An aliquot of the samples were then transferred into 100-mL vessels and treated with overpressure of 0.2 mPa for 2 min. In consequence, gas vesicles, and in some cases, cells of most cyanobacteria (e.g. *Aphanizomenon*) collapsed, whereas *Microcystis* cells were still intact and buoyant. After the pressure treatment, the suspension was

centrifuged (3 000 g) to separate *Microcystis* colonies from the destroyed cells of other cyanobacterial spp. BG-11, designed by M.M Allen was used as growth media. The liquid BG-11 nutrient medium containing 17.65 mM $NaNO_3$, 0.18 mM $K_2HPO_4.3H_2O$, 0.30 mM $MgSO_4.7H_2O$, 0.25 mM $CaCl_2.2H_2O$, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM EDTA (ethylenediamine tetra-acetic acid, disodium magnesium), 0.19 mM Na_2CO_3, 0.05 mM H_3BO_3, 9.15 mM $MnCl_2.4H_2O$, 0.77 mM $ZnSO_4.7H_2O$, 1.61 mM $Na_2MoO_4.2H_2O$, 0.37 mM $CuSO_4.5H_2O$ and 0.17 mM $Co(NO_3)_2.6H_2O$ dissolved in 1L distilled water. Freshly poured petri plates were left to cool, solidify, and dry in a laminar flow hood sterilized by UV irradiation. The warm plates were stacked on top of each other to prevent the formation of condensation below their covers. The medium was left to dry at 37 ºC for 24 hr.

When the agar medium was solidified in Petri dishes a bacterial loop was mainly employed in the initial stages of isolation. With the loop, heated in the flame of a bunsen burner and cooled at one edge of the agar plate, the crude cyanobacterial material of the unicellar and axenic strains deposited, was densely spread over the first quarter of agar medium. The loop was then flamed again, cooled and used to perform several parallel streaks traversing at an angle of 90° to the previously spread material. The incubation took place at 25 ºC under continuous illumination of approximately 60 qmol photons/m^2/s^1 from cool white fluorescent tubes for 7 to 10 days (first culture). When colony formation was observed, the *Microcystis aeruginosa* cells in the colonies were identified using Komárek's (1958) criteria of colony morphology and cell diameter. These *Microcystis* cells were than transferred by a sterilized toothpick to fresh agarose medium and incubated again (second culture).

Longterm first culture has been avoided due to the possibility for contamination increase by bacteria and fungi. The cells in fully propagated colonies in the second culture were inoculated into BG-11 medium (5mL per tube) and cultured for 1 to 2 weeks. Then, the culture was examined by a phase-contrast microscope to determine whether any contamination had occurred. If contamination was observed, the culture was returned to the first step of the isolation procedure. The cells from the cultures that were confirmed to be contamination free by microscopic examination were encircle by an autoclaved toothpick and an incision through the agar was made. The tiny piece of agar bearing the microcolony of *M. aeruginosa* was inoculated into 100 mL of BG-11 medium in a 250-mL Erlenmeyer flask and then cultured. The purity of the resulting cultures was verified weekly by the absence of bacterial growth on TYG agar and TYG broth [tryptone (Difco) 5.0 g; yeast extract (Difco) 2.5g; glucose, 1.0 g per liter].

The final proof of purity was verified by microscopic examination. Cultures of *Microcystis aeruginosa* were harvested at the end of exponential growth phase (three weeks) by centrifugation at 6 000 g for 10 min at room temperature. The cultures were then freeze-dried and stored at –20 °C.

6.2.2 DNA Extraction

Genomic DNA was isolated from a freeze-dried culture according to a modified method of Raeder and Broda (1985). One volume extraction buffer (200 mM Tris-HCl (pH 8.00), 150 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol, 1 % (w/v) Polyvinylpyrrolidone, PVP) was added to each gram of freeze-dried culture, and homogenized in the presence of washed sand, thereafter the

homogenate was placed at 60 °C for 10 min. The homogenate was then centrifuged at 6 000 g for 15 min. After removal of the supernatant, the extraction was further purified by the addition of equal volumes of chloroform:phenol (1:1). The resulting mixture was vortexed and centrifuged again at 6 000 g for 15 min, where after the upper layer was carefully removed. This was followed by a DNA precipitation step using two volumes of ice-cold absolute ethanol and stored at –20 °C for at least 1 h. The DNA mixture was centrifuge at 6 000 g for 15 min, and the resulting pellet was washed with 70 % ethanol. The ethanol wash-step was repeated three times, and dried after removal of the liquid. The resulting DNA was resuspended in distilled water and stored at –80 °C until further use (Oberholster 2004).

Quantification of the DNA concentrations was done through spectrophotometric measurements at absorbances of 260 and 280 nm, using a Beckman DU650 Spectrophotometer. The DNA quality was also assessed and the concentration determined by visualisation under UV light, on 1 % TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) agarose gels containing ethidium bromide (Sambrook *et al.* 1989).

6.2.3 AFLP analysis

To determine the usefulness of AFLP analysis to discern between the individual strains, as well as their geographical origin, AFLP analysis was conducted using the the IRDye^{TM} Fluorescent AFLP® Kit (LI-COR Biosciences, Lincoln, USA) following the manufacturer’s instructions. Restriction endonucleases were done with primer combinations *Eco*R1/*Mse*1. For this, genomic DNA (75 ng) was incubated for 2 h at 37 °C with 1.25 U of *Mse*1, 1.25 U of *Eco*R1, 1 U of T4 DNA ligase, 40 pmol of *Mse*1 adapters and 10 pmol *Eco*R1 adapters. This reaction was done in a volume of

50 µL restriction–ligase buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM dithriothreitol (DTT), 0.1 mM EDTA, 50 % (v/v) glycerol, 0.15 % (v/v) Triton X-100 and 200 ng/µL BSA. Termination of the reaction was done by heating at 70 ˚C for 15 min, where after it was placed on ice. For adaptor ligation, 25 µL of the Adapter mix (containing *Mse*1 adaptors and *Eco*R1 adaptors, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, and 50 mM K-acetate, and 1 U of T4 DNA ligase) was added followed by incubation at 37 °C for 3 h. An aliquot (10-µL) of the adapter-ligated DNA was diluted (1:10) with TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) to serve as template in the preselective amplification PCR. The remaining portion was used to verify whether the digestion was complete (Oberholster 2004).

The preselective amplification reaction contained 2.5 µL of adapter-ligated DNA (diluted 1:10), 2.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2.5 µL of 10 x PCR reaction buffer (Roche Molecular Biochemicals), 15 mM $MgCl_2$, 500 mM KCl and 100 µM of IRDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 and *Mse*1 primers (containing dNTPs) with every selective nucleotide, in a total volume of 25.5 µL. The amplification procedure consisted of twenty cycles of 30s at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and soaked at 4 °C. The selective PCR contained 2 µL of the diluted (1:10) product of the preselective PCR, 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2 µL of 10 x Taq DNA polymerase buffer (Roche Molecular Biochemicals), KCl and $MgCl_2$ as mentioned above, and 100 µM of IRDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 and *Mse*1 primers (containing dNTPs) with every selective nucleotide, in a total volume of 11 µL. Eight *Eco*R1-*Mse*1 primer pairs (LI-COR Biosciences, Lincoln,

USA) were used for selective amplification (Table 6.2). The first amplification cycle was carried out for 30s at 94 °C, 30s at 65 °C and 1 min at 72 °C. At each of the following 12 cycles, the annealing temperature was reduced by 0.7 °C per cycle. The last 23 cycles of annealing were carried out at 72 °C for 1 min; and then soaked at 4 °C (Oberholster 2004).

6.2.4 Gel electrophoresis and scoring

An equal volume of loading solution (LI-COR Biosciences, Lincoln, USA) was added to each selective amplification reaction. Samples were denatured at 95 ºC for 3 min and placed on ice for 10 min before loading. A volume of 0.8 µL was loaded with an 8-channel syringe (Hamilton, Reno, Nevada) onto 25-cm 8% Long Ranger gels (BMA, Rockland, ME, USA). Electrophoresis and detection of AFLP fragments were performed on LI-COR IR2 (model 4200S) automated DNA analysers. The electrophoresis parameters were set to 1 500V, 40mA, 40W, 50 ºC, and a scan speed of 3. The run-time was set to 4 h and gel images were saved as TIF files for further analysis.

The gel images were scored using a binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively. Semi-automated scoring was performed with AFLP Qauntar Pro (Version 1,0, LI-COR) and followed by manual editing to make adjustments to the automated score where necessary. A locus was scored as polymorphic when the frequency of the most common allele (band present or absent) was less than 0.97 (absent or present in at least two individuals). Bands with the same

mobility were considered as identical products (Waugh *et al*. 1997), receiving equal values regardless of their fluorescence intensity.

6.2.5 Data analysis

Polymorphic bands scored as plus (+) and minus (-) and converted to 1 and 0 were compiled in a data matrix. The data matrix was used to perform cluster analysis on the basis of the average linkage method, known as the Unweighted Pair Group Method using Arithmetic averages (UPGMA) and PAUP 4.0 software (Hintze 1998). The average polymorphic information content (PIC) was calculated across each primer combination according to Riek *et al*. (2001) as follows:

$$PIC = 1-[f^2+(1-f)^2]$$

Where f is the frequency of the marker bands in the data set. Marker index (MI) was determined by multiplying PIC values with percentage polymorphism for each primer combination (Lübberstedt *et al*. 2000). The raw data matrix was used to estimate genetic similarities among different geographical cyanobacterial strains. Estimates of genetic similarity between all pairs of strains were calculated in the form of dissimilarity and expressed as Euclidean genetic distance (Jacoby *et al*. 2003). The 'goodness of fit' of the clustering to the data matrix was determined by calculating the cophenetic correlation coefficient between the dissimilarity matrix and the cophenetic matrix derived from the dendrogram (Sneath & Sokal 1973; Sneath 1989).

6.3 Results

6.3.1 Fast screening of AFLP primer combinations

After screening 20 primer combinations on a subset of strains using either IRDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 primers, eight IRDye700^{TM}-labeled *Eco*R1 primer pairs were selected for analysis (Table 6.2).

Table 6.2 Degree of polymorphism and average polymorphism information content (PIC) and marker index (MI) for the eight AFLP primer combinations used to analyse the 23 strains.

No	Primer combination	Total number of bands	Number of polymorphic bands	% of polymorphic bands	PIC	MI
1	*Eco*R1-ATC / *Mse*1-CCA	53	52	98.11	0.287	28.156
2	*Eco*R1-AAA / *Mse*1-CCC	18	17	94.44	0.293	27.671
3	*Eco*R1-ACG / *Mse*1-CCC	34	33	97.05	0.324	31.444
4	*Eco*R1-ACG / *Mse*1-CAC	19	18	94.73	0.363	34.387
5	*Eco*R1-ACT / *Mse*1-CAC	47	45	95.74	0.342	32.743
6	*Eco*R1-AT / *Mse*I-CG	28	25	89.28	0.326	29.105
7	*Eco*R1-AT / *Mse*1-CT	43	42	97.67	0.360	35.161
8	*Eco*R1-CC / *Mse*1-CT	34	34	100.00	0.397	39.700
	Total	276	266	NA	NA	
	Mean	34.5	33.25	95.87	0.337	32.308

Primer pair *Eco*R1-ATC/*Mse*1-CCA amplified the largest number of bands (53), but the lowest PIC (0.287) and a MI of 28.156. Primer pair *Eco*R1-AAA / *Mse*1-CCC amplified the lowest number of bands (18) and had the lowest MI (27.671). While primer pair *Eco*R1-CC / *Mse*1-CT had the highest PIC (0.397) and MI (39.700). The generated fingerprints were evaluated for repeatability and overall clearness of the banding pattern. The number of informative fragments was also taken into account (Fig. 6.2). We found no significant difference in MI through the inclusion of primer

pairs with two rather than three selective nucleotides, since the mean MI of the primer pairs with three nucleotides was 30.881 ± 3.506 and the MI of primers with two selectives was 34.655 ± 5.044. A total of 276 bands were amplified from the eight primer combinations, of which 266 were informative, 10 non-informative, with an average of 95.87 polymorphic bands per primer combination.

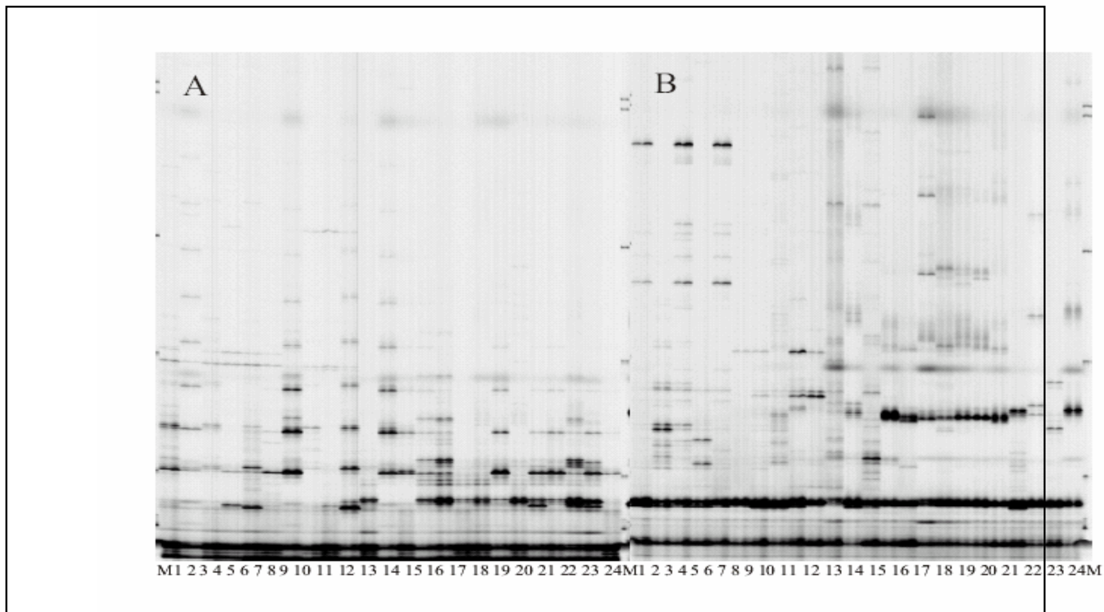

Figure 6.2 AFLP banding patterns generated using primer combinations E*co*R1-CC/*Mse*1-CT (A) and *Eco*R1-ATC/*Mse*1-CCA (B). M = 100 bp ladder marker; 1 = NIES88; 2 = NIES89; 3 = NIES90; 4 = NIES99; 5 = NIES299; 6 = PCC7806; 7 = CCAP1450/1; 8 = SAG1; 9 = UV027; 10 = PCC7813; 11 = UP01; 12 = UP02; 13 = UP09, 14 = UP04, 15=UP04, 16 = UP10, 17 = UP13, 18 = UP15, 19 = UP26, 20 = UP37, 21 = UP38, 22 = UPUS1, 23 = UPUS2, 24 = UP06.

6.3.2 Genetic diversity as defined by AFLP fingerprinting

The genetic relationship among all the *Microcystis aeruginosa* strains based on the combination of data obtained with the eight primer combinations is represented in the dendrogram (Fig. 6.3).

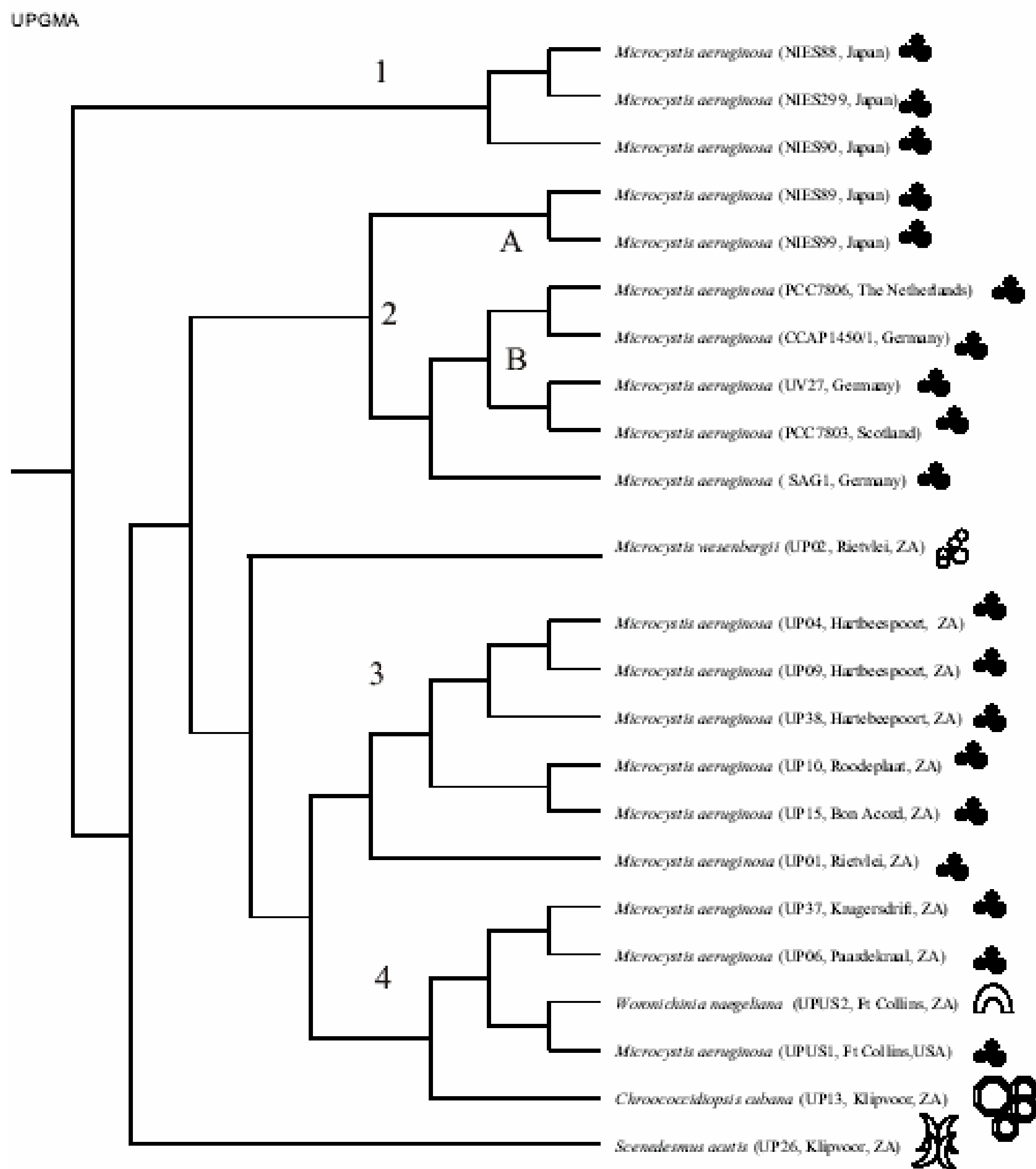

Figure 6.3 Combined cluster analysis derived from AFLP analysis of 23 *Microcystis aeruginosa* and outgroup strains using eight AFLP primer combinations.

The dendrogram consists of four clusters. Cluster one contains NIES88, NIES299 and NIES90 basal to this group. The mean character difference between the NIES88 and NIES299 strains were 0.163, while they differ in pairwise distance from NIES90 with a mean of 0.243. The mean character difference of 0.163 is also the lowest obtained mean value in the data set. The largest mean character differences of 0.558 were obtained between the strains *Scenedesmus acutis* (UP26) and *M. aeruginosa* (NIES88), and *M. wesenbergii* (UP02) and *M. aeruginosa* (NIES90), respectively.

In cluster 2 there are two groupings. In cluster 2A, the NIES strains 89 and 99 group together with a mean character difference of 0.243, while in 2B strains PCC7806 and CCAP1450/1 fall into a group with a mean character difference of 0.207, and UV27 group with PCC7813 (mean character difference of 0.192), with SAG1 basal to this group.

Cluster 3 consists exclusively of *Microcystis aeruginosa* strains from the geographical area of Gauteng and North West provinces in South Africa, and includes strains UP04, UP09, UP38, UP09 and UP15, with UP01 basal to this group. The strains from Hartbeespoort reservoir (UP04, UP09 and UP38) also group together, while UP10 (Roodeplaat) and UP15 (Bon Accord) forms a subgroup. The geographical distances between the Hartbeespoort reservoir and Bon Accord and Roodeplaat reservoirs are 36.6 km and 53.8 km, respectively (Table 6.3). UP01 was collected from the Rietvlei reservoir that is located in a south-eastern direction to all the above reservoirs. The mean character difference between UP01 and the Hartbeespoort strains was 0.361.

Table 6.3 Geographical distance in kilometers between the reservoirs in Gauteng and North West Provinces, South Africa.

Reservoir	Klipvoor	Hartbeespoort	Bon Accord	Rietvlei	Roodeplaat
Klipvoor	0.0	65.9	66.6	96.5	78.4
Hartbeespoort	65.9	0.0	36.3	48.1	53.8
Bon Accord	66.6	36.3	0.0	30.7	18.1
Rietvlei	96.5	48.1	30.7	0.0	30.0
Roodeplaat	78.4	53.8	18.1	30.0	0.0

In cluster 4, UP37 and UP06 of the geographical area of the Free State and Western Cape province of South Africa group together with a mean character difference of 0.275. The strains UPUS1 and UPUS2 of Colorado U.S. fall in a separate group, while strain UP13 is basal to cluster 4.

6.4 Discussion

AFLP fragments have been used to unravel cryptic genetic variation for a wide range of taxa, including plants (Mackill *et al.* 1996), fungi (Majer *et al.* 1996, 1998) and bacteria (Huys *et al.* 1996), which have previously been impossible to resolve with morphological characters. In the present study, complex AFLP banding patterns were obtained. Janssen *et al.* (1996) have showed that the choice of the restriction enzymes, and the length and composition of the selective nucleotide will determine the complexity of the final AFLP fingerprint. Primer selectivity is good for primers with one or two selective nucleotides in simple genomes such as fungi, bacteria and some plants, and still acceptable with primers having three selective nucleotides, but is lost with the addition of a fourth nucleotide (Vos *et al.* 1995). We used a combination of primers with two and three selective nucleotides on 23 *Microcystis aeruginosa* and outgroup strains, and a total of 276 bands were amplified, constituting

95.87 % informative bands and 4.13 % monomorphic bands (Table 6.2). However, we did not gain advantage in number of amplified loci when using two rather than three selective primers sets.

In the dendrogram, the Japanese strains (NIES88 NIES90, NIES299) group separate from the other NIES strains (cluster 1), with NIES88 and NIES299, genetically closest to each other, while NIES strains 89 and 99 group together with the European strains (cluster 2). In cluster 3, all the *Microcystis aeruginosa* strains of Gauteng and North West province of South Africa group together, except for the outgroup strain *Chroococcidiopsis cubana* from Klipvoor dam. This phenomenon may be due to the fact that geographical distance between the *Microcystis* strain locations in cluster 3 is close and are located in the northern geographical region of South Africa (Table 6.3).

The position of the UPUS1 and 2 strains in their respective cluster is surprising, since these strains originated from Colorado, U.S. However, they group together with the unrelated strains from the central and southern geographical areas of South Africa (i.e., the Western Cape and the Free State provinces), but not with the *Microcystis* strains from the Gauteng and North West provinces or the European strains. There are two plausible explanations for this observation. Firstly, this occurrence is most likely the result of a reflection of ease of dispersal; yet for the majority of species there is little information on their mechanism of transport (Round 1981). Among the factors shaping the geographical distribution of cyanobacterial species, temperature is usually considered to be of prime importance. Other factors that also play an important role are migratory barriers for example oceans and mountain ranges, as well as passive dispersal agents involving water, air and different animals. The probability of

successful dispersal to other geographical areas depends strongly on the effectiveness of the carrier, and the ability of cyanobacteria to tolerate the transport conditions (Kristiansen 1996a, b). Water beetles and aquatic mammals may be effective over a distance of a few kilometres, whereas dragonflies have been shown to transport viable algal material almost 1 000 km. Atkinson's (1980) reported in his experiments that viable remains of *Asterionella* was found in the faeces of waterfowl after a maximum of 20 hours, perhaps corresponding to a flight of 220 km. Atkinson (1980) has also shown that transport by the digestive tract of birds may be more efficient because desiccation is ruled out. Specialised life cycle forms for example spores, cysts and akinetes, as well as thick, resistant cell walls like in the case of *Microcystis* spp. will better survive longer transport, either externally or internally. Many ducks migrate up to 4 800 km, but with many stops. Nonstop flight distances of the order of 3 200 km in 48 hours have been noted for the white-fronted goose (*Anser albifrons*) (Owen & Black 1990). For airborne transport, it is very difficult to give maximal distances. Since viable *Melosira* were found at 3 000 m altitude, this dust containing diatom frustules can certainly be blown across the Atlantic Ocean (Kristiansen 1996b) and with it, possible some species of cyanobacteria.

A second explanation for the grouping of UPUS1 and 2 together with the South African strains, rather than with the European and NIES strains, may be due to the fact that the so called traditional cultured strains PCC, NIES, CCAP1450/1, UV and SAG1 are decades in prolonged subcultures. In contrast, the UP and UPUS are much shorter in subculture and thus 'wild-type' strains. This phenomenon is most likely due to the fact that although nearly all populations of cyanobacteria from different locations differ to some degree from each other, they may stabilize in longterm

cultures (Waterbury & Rippka 1989; Kato & Watanabe 1993; Van der Westhuizen & Eloff 1982). Carr (1999) has argued convincingly about the bias introduced to research by use of strains selected for their ability to grow exponentially. There exists abundant evidence that morphological changes take place during prolonged subculture, with almost as much evidence for physiological changes (Doers & Parker 1988). Fray (1983) stated that records of mutations (i.e., permanent changes of the genome) are well substantiated in laboratory cultures. These include longterm subculture strains that fail to differentiate heterocysts and to synthesize nitrogenase (*Anabaena variabilis*); which lack the ability to produce akinetes (*Anabaena cylindrical*); which are incapable of forming gas vesicles (*Anabaena flos-aquae*); synthesizing toxin (*Microcystis aeruginosa*) or depositing sheath material. Such mutants generally outgrow the 'wild type' strains because energy previously invested in the formation of structures, which confer no advantage in particular conditions, can be diverted to cell growth.

We have also noted two outgroups in cluster 4, namely *Chroococcidiopsis cubana* (UP13) belonging to the division Cyanophyta (Fig. 6.1D), which cells are solitary or aggregated in irregular groups, enveloped by thin, firm, colourless sheaths. This species is non-heterocystous but able to fix N_2. The cells are spherical, oval to irregular rounded of varying sizes between 1.5-20 µm in diameter and are usually attach to stony substrata in the aquatic habitat. The other outgroup in this cluster is *Woronichinia naegeliana* which comprises of spherical or irregular, free-living colonies surrounded by a fine, colourless mucilage (Fig. 6.1B). Cells are at or within the ends of mucilaginous stalks and radially arranged with dimensions of (1)2.5-7 x 1-4(5) µm. Stalks are densely packed, causing radial lamellation, but sometimes are

diffuse near the center. Of the 15 described species, 3 contain gas vesicles of which *Woronichina naegeliana* is one, and may form cyanobacterial surface blooms in eutrophic North American waters (Komárek & Anagnostidis 1998). The other outgroups that don't fall in any cluster is (UP3) *Microcystis wessenbergii* (Fig. 6.1A) and the green algae (UP26) *Scenedesmus acutis* which belong to the division Chlorophyta. Colonies of *Scenedesmus* are 2-4-8-16-32 celled, flattened, with long axes of cells parallel, laterally adjoined and arranged in single linear or alternating series. Cells are ellipsoidal or tapering toward each end, while the cell wall is smooth and spines are absent (Shubert 2003).

Nielan *et al.* (1995) assessed the usefulness of AFLPs and restriction fragment length polymorphism (RFLP) analyses on members of the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospemopsis*, *Nodularia*, *Nostoc* and *Planktothrix* with regard to cyanobacterial systematics. In their study, they were able to discern the individual strains when using AFLP analysis, but not always when using RFLPs. They observed delineation of the *Microcystis* strains with regard to geographical origin when using both AFLP and RFLP analyses, but the technologies were not able to discriminate between strains on the basis of their levels of toxicity. But more importantly, they found no relationship between the PCR amplicons and restriction fragment profiles obtained with the respective genera and the taxonomic placement of the respective genera in cyanobacterial systematics.

In view of the present study, AFLP analysis is useful for the identification of genetic diversity of geographical unrelated strains and analysis of *Microcystis aeruginosa* strains. AFLPs seem to overcome the major pitfalls present in other PCR based

methods, e.g. DAF or RAPD analysis, and appear to be as reproducible, heritable and intraspecific as RFLPs (Law *et al*. 1998). The use of the AFLP fingerprinting method resulted in a high degree of discrimination and identification of geographically unrelated *Microcystis aeruginosa* strains, and was found useful and practical. The results from this study is concurrent with the results by Nielan *et al.* (1995) that AFLP analysis is not suitable if evolutionary phylogeny is the objective, since the data did not support the taxonomic placing of the genera, but rather the geographical origin.

Acknowledgements

The authors would like to express their sincere gratitude to Karin van Ginkel (Department of Water Affairs and Forestry) and Leanne Coetzee (Tshwane Metro Council) for the collection of some of the strains. Also, we wanted to thank the National Foundation of Research, and the Water Research Commission, South Africa for provision of funding. We especially want to thank Dr. Jiří Komárek for his assistance with the identification of some of the strains.

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Summary

In recent years ecological systems are affected by various natural and anthropogenic disturbances. These disturbances not only affect resource availability, physical and chemical environments, but they also disrupt ecosystem and community structure (Connell 1978, Resh *et al*. 1988). The impacts of human disturbances on stream ecosystems (e.g., organic enrichment, eutrophication, heavy metals, pesticides and petroleum pollution) have become more and more ubiquitous. These disturbances play an importance role in the shaping of fresh water ecosystems (Petersen *et al*. 1987). Municipal and industrial wastes, which are important pollutants of urbanized rivers and lakes, results in organic enrichment and metal pollution (Mason 1996). In addition, an increase in nutrient concentrations are a serious and well known consequence of a greater human presence within a watershed because municipal wastes and fertilizers are also significant nutrient sources from urban storm-runoff (Mason 1996). Urban runoff and industrial effluents may contain organic and inorganic substances potentially toxic to aquatic biota, whose identification is a difficult task that involves expensive and complex analytical techniques. At the present time there are approximately 70 000 chemicals in use or being distributed throughout the environment, and an additional 500-1000 are added each year. Although not all these chemicals are toxic, merely keeping up-to-date information on the possible toxicity of so many compounds is a prodigious task (Postel 1987). A further problem is the interaction that may occur once potentially toxic substances are mixed, which may result in highly toxic effluents. The complexity of ecosystems makes it difficult to estimate the explicit effects of given physicochemical factors on the structural and functional attributes. It is becoming more important for the development of methods which can analyze patterns of biotic communities and

assessing the relative importance of multiple environmental variables on species assemblages.

The introduction of urban runoff pollutants such as nutrients and organic matter into the aquatic system of Sheldon Lake a year after restoration has set off a complicated series of biological and chemical reactions. These changes were brought about acceleration of the phytoplankton community during the summer of 2004 causing cultural eutrophication. Cyanobacterial species which are frequently associated with eutrophic systems dominated from June to September although the lake where artificially mixed. The artificial mixing alone were insufficient to cause a transition to a well-mixed system but at the end of summer, high-wind and storm events change the lake from a incomplete to a complete mixed water-body. A year after the bottom sediment removal for restoration purposes and to increase water detention, there are notably low abundance of macrophytes at all the sampling sites and submerged vegetation had failed to colonize the main basin. This phenomenon had led to consumer resistance among smaller organisms for e.g., *Bosmina* sp. that used the cyanobacterial surface bloom as refuge from grazing by planktivorous fish because of the lack of submerged vegetation. Furthermore the juvenile blue gill sunfish (*Lepomis macrochirus*) had changed their habitat choice to the littoral zone in response to presence of big mouth bass (*Micropterus salmoides*) and the lack of submerged vegetation in the pelagic zone. The highest density of invertebrates where observed in the soft sediment at an inlet of one of the urban runoff pipes. These species where the chironomid larvae of the genus *Chironomus* and Oligochaete worms which is also associated with organic pollution lotic systems and reflect the dominant functional feeding group collector-gatherers. During the summer cyanobacterial surface blooms

were sampled and, microcystin molecular markers were used for the detection of toxic cyanobacterial strains in environmental samples. The *Microcystis* spp. strains from Sheldon Lake were analyzed by polymerase chain reaction with oligonucleotide primers that derived form the *mcy* gene cluster that is involve in microcystin synthesis. The presence of the *mcy* gene cluster in the analyzed strains indicates that the strains have the genetic potential to produce microcystin. The toxicity of the strains was also confirm with a protein phosphatase inhibition and ELISA assays. This data is of economic and public health value since it is be able to detect early stage blooms of toxic cyanobacteria, and *Microcystis* in particular, especially if it is on a sufficiently timely basis for municipalities and recreation facilities to implement a response plan for example in the case of Sheldon Lake. We also used Amplified fragment length polymorphism markers, which is based on the selective amplification of genomic restriction fragments by PCR, to bring into genetic "context" the collected strains from Colorado to other strains used in the study. We further differentiate between a *Microcystis aeruginosa* strain of Sheldon Lake, United States and geographical unrelated strains from South Africa, Europe and Asia. The study clearly demonstrates the superior discriminative power of AFLP towards the differentiation of geographical unrelated *Microcystis aeruginosa* strains that belong to the same species.

In the case of the Cache la Poudre River the presence of certain species and their relative abundance were used as well as biomarkers to measure the degree of coal tar contamination. Because of the numerous routes of exposure we found that, almost any species may be affected by coal tar. The dominance by a single phytoplankton taxon that was significantly higher at the contaminated sampling sites, than in the case of the

reference areas were most probably due to environmental stress of coal tar residue on the other species. The Australia River Assessment system method which is based on the presence and absence of aquatic macroinvertebrates, compares to the number of animals found at a minimally degraded reference site, was used in the survey study. We observed that sampling sites 2, 4, 5 and 7 were severely degraded with none of the expected macro-invertebrate families found, which show a good parallelism with the results of the battery bioassay and also indicated the areas of highly contaminated sediment. Although we did not attempt in this study to measure toxic residue concentrations of the coal tar, we found that the biotest battery is useful, sensitive and inexpensive tool to detect toxicants in the environment.

To conclude, it is clear from the study that anthropogenic activities impacted negatively on both studied aquatic ecosystems, resulting in severe deterioration of the water quality in the Cache la Poudre River, and in the case of Sheldon Lake eutrophication and the associated bloom formation of toxic cyanobaterial spp., and thus, management strategies have to be developed to alleviate the severity of impact. To address the raised issue, several recommendations to improve management strategies for both water systems are made. In the case of the urban Sheldon Lake, it is suggested that a wetland system should be constructed to "filter" the inflow water from urban runoff and the Cache la Poudre River before it enters the lake. This will improve the quality of the inflow water to the lake, since the ability of wetlands to remove nutrients from urban runoff and sewage effluents is well documented (Wood 1994). Typically shallow wetlands allow for ready trapping of particles by the sediments and nutrient uptake by the aquatic vegetation and sediment microbes. However, two major drawbacks of routing flow through a constructed wetland is

firstly, the increased water loss due to evapotranspiration. It is estimated that as much a third of the effluent flow would be lost. Secondly, a constructed wetland may raise summer water temperature above those of the inflowing Cache la Poudre River, known as thermal mediation, affecting the intrusion dynamics in the receiving lake (Andradóttir & Nepf 2000). In the case of the Cache la Poudre River, the environmental laws should be greatly enforced, since the deterioration in the river ecosystem is mainly due to pollution and waste disposal. In the US about 95% of industrial and other hazardous waste is usually deposited of on site where it is generated (Postel 1987), making the Cache la Poudre a potential target for these practices, as was shown in my case study. It is further recommended that the battery of bioassays that was developed in the study be utilized to monitor the "health" in both the river and proposed constructed wetland ecosystems on a regular basis.

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